Regulation of Epidermal Growth Factor Receptor Signaling during Oxidative Stress

Regulatie van Epidermale Groeifactor Receptor Signalering tijdens Oxidatieve Stress

(met een samenvatting in het Nederlands)

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Abbreviations

AGF	agialofetuin
AGF-Cy3	Cy3-conjugated AGF
AP	adaptor protein
BSA	bovine serum albumin
CAT	catalase
CHL	chicken hepatic lectin
CSLM	confocal scanning laser microscopy
Cys	cysteine
DMEM	Dulbecco's modified Eagle's medium
DSP	dual specificity phosphatase
DTNB	5-5'-dithiobis (2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGF-biotin	biotin-conjugated EGF
EGF-Rhod	tetramethyl-rhodamine-conjugated EGF
Eps	EGF receptor pathway substrate
GAP	GTPase activating protein
GHR	Growth Hormone Receptor
GPX	glutathione peroxidase
GRD	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HUB	HA-tagged ubiquitin
kDa	kilodalton
LDH	lactate dehydrogenase
LMW	low molecular weight
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase or ERK kinase
NEM	N-ethylmaleimide
OPD	*O*-phenylene-diamine dihydrochloride
PBS	phosphate-buffered saline
PBSgluc	PBS supplemented with 5 mM glucose
PBS-0	PBS without 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$
PDGF	platelet-derived growth factor
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PPD	p-phenylene-diamine
PTB	phosphotyrosine-binding
PTP	protein tyrosine phosphatase
PY20	anti phosphotyrosine antibody
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RPTP	receptor protein tyrosine phosphatase
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel-electrophoresis
Ser	serine
SH	sulphydryl
SH2	Src homology 2
SOD	superoxide dismutase
Strepta-vidin-PO	horseradish peroxidase-conjugated streptavidin
TCA	trichloroacetic acid
Thr	threonine
Tyr	tyrosine

Chapter 1

General Introduction

Based on: Oxygen Free Radicals and Cell Signaling
Renate de Wit, Johannes Boonstra, Arie J. Verkleij, Jan Andries Post
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Introduction

During oxidative stress, cells are exposed to elevated levels of oxygen free radicals, which can form a threat to normal function or even to life. Oxygen free radicals are produced during both normal and pathological cell metabolism and have been implicated in processes as aging and carcinogenesis, suggesting that oxygen free radicals can affect cell proliferation and differentiation. Important components of the regulatory systems that coordinate cell growth and differentiation are polypeptide growth factors. Growth factors initiate signaling pathways via plasma membrane receptors such as the tyrosine kinase and the integrin receptors. Binding of the ligand induces activation of the receptor, followed by the recruitment and activation of various intracellular signaling molecules. This finally leads to the activation of transcription factors, altered gene expression and a cellular response such as proliferation, migration, differentiation or apoptosis.

In the last decade, many studies have shown that oxygen free radicals that are either added extracellularly or generated intracellularly in response to growth factors, are capable of interfering with signal transduction pathways. One of the mechanisms through which free radicals activate signaling molecules is by the reversible inactivation of protein phosphatases, through oxidation of essential sulphydryl groups within their active site cysteines. Since many proteins contain sulphydryl groups that are involved in their enzymatic activity, protein-protein interactions or tertiary structure, protein oxidation might result in disturbed signal transduction and cellular functioning. Therefore, it is important to gain insight in the effects of oxidative stress on signal transduction pathways, on the regulation of these signaling pathways and in the mechanisms underlying these effects.

Oxidative Stress

Oxygen and oxygen free radicals

The earth's atmosphere consists for 21% of oxygen, which allows the survival of oxygen-requiring organisms by aerobic life forms. The human body consumes oxygen and crucial body functions cannot last longer than a few minutes without a fresh supply of oxygen. As a consequence of utilization of oxygen, however, oxygen free radicals and reactive oxygen species (ROS), which include both oxygen free radicals and molecules that can give rise to oxygen free radicals, are produced during normal cell metabolism. Free radicals may be defined as any chemical species that contains one or more unpaired electrons and is capable of independent existence.[1] These unpaired electrons make oxygen free radicals highly reactive and reaction with nonradicals may form new radicals, which may result in chain reactions of free radical formation.[2] In cells, free radicals can react with organic compounds and cause damage to DNA, lipids and proteins. Exposure of cells to elevated levels of oxygen free radicals is referred to as oxidative stress, which is due to an imbalance

between production of free radicals and the ability of antioxidant defense systems to cope with these radicals. Oxidative stress and the damage that it causes have been implicated in both natural processes, such as aging, and pathological processes, including atherosclerosis, cancer, neurological degeneration such as Alzheimer's disease, schizophrenia, and autoimmune disorders such as arthritis.[3-8]

Generation of oxygen free radicals in cells

The mitochondrial electron transport chain generates adenosine 5'-triphosphate (ATP) via oxidative phosphorylation and is considered as the main source of cellular ROS. Reduction of oxygen to water in the electron transport chain can proceed by at least two pathways. First, cytochrome oxidase is capable of reducing oxygen to water by a tetravalent reduction without the production of any intermediates.[9] The second pathway proceeds univalent in which several intermediates are formed, such as superoxide ($O_2^{\cdot -}$) and hydrogen

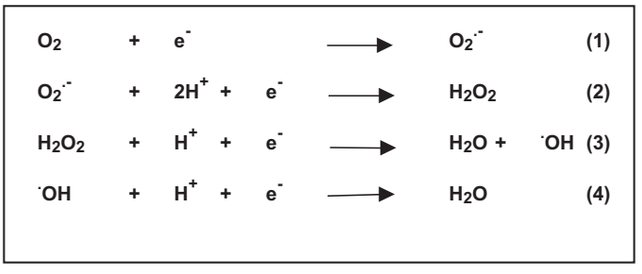

Fig.1. Univalent reduction pathway of molecular oxygen to water.

peroxide (H_2O_2) (Fig. 1).[10,11] Inefficient removal of these intermediates will, especially in the presence of a transition metal such as iron, result in the formation of the highly reactive hydroxyl radical (·OH), which might be responsible for most of the oxidative damage in biological systems.[2,12-15] Other cellular sources of ROS are peroxisomes that generate H_2O_2 by degradation of amino acids and fatty acids during the β-oxidation.[16]

Oxygen free radicals are also produced by macrophages and neutrophils, which combat microorganisms by destroying them with an oxidative burst of a powerful mixture, consisting of various reactive oxygen species, including H_2O_2 and $O_2^{\cdot -}$.[17,18] The most important enzyme complex responsible for radical production in these cells is a membrane-bound NADPH oxidase, which catalyses the reduction of oxygen to $O_2^{\cdot -}$.[19] Moreover, nitric oxide (NO·) is synthesized by inflammatory cells and has the potential to react with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$), which is a potent cytotoxic species. Through leakage of radicals by phagocytes, inflammation may cause damage to lipids, proteins and DNA of surrounding cells.[20] Therefore, chronic infections can contribute to the carcinogenic process through the

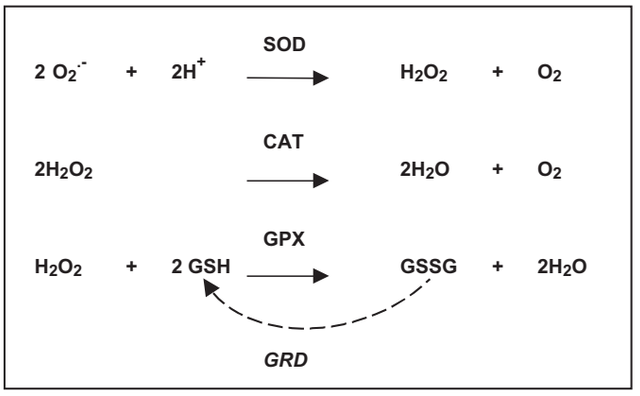

Fig. 2 Antioxidant enzymes. Superoxide dismutase (SOD) reduces $O_2^{\cdot-}$ to H_2O_2 and O_2. Both catalase (CAT) and glutathione peroxidase (GPX) subsequently convert H_2O_2 to $H_2O + O_2$ or H_2O respectively. Glutathione reductase (GRD) ensures a constant supply of reduced glutathione (GSH) by reduction of oxidized glutathione (GSSG).

formation of oxygen free radicals.[21]

In addition to the sources as mentioned above, oxygen free radicals can also be generated by ionizing radiation, ultraviolet (UV) light, ozone, the metabolism of certain xenobiotics and cigarette smoke.[22-24]

Cellular antioxidant defense mechanisms

Cells are equipped with numerous enzymes and compounds that function to protect the cell from oxidant damage.[25,26] Under normal conditions, cellular oxygen free radicals are kept at low levels through the coordinated action of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Fig. 2).[27,28] SOD, which is predominantly present in mitochondria and in the cytoplasm, uses one $O_2^{\cdot-}$ molecule to reduce another $O_2^{\cdot-}$ molecule to H_2O_2 and O_2. H_2O_2 can subsequently be converted to H_2O and O_2 by either GPX, present in the cytoplasm and mitochondria, or CAT, which is localized in peroxisomes. Glutathione reductase (GRD), in a reaction requiring NADPH, catalyzes reduction of GSSG to replenish reduced glutathione (GSH). GSH in its turn functions as electron donor, resulting in the formation of oxidized glutathione (GSSG). The intracellular GSH:GSSG is crucial in maintaining redox system homeostasis and this appears critical to normal cellular processes such as regulation of cell proliferation and activation of specific genes.[29]

Endogenous nonenzymatic antioxidants include a variety of lipophilic and hydrophilic molecules or protein components, that act as oxygen free radical scavengers.[28]

Scavenging can be accomplished by the reaction of sulphydryl (SH) groups in these molecules or proteins with free radicals.[30] Moreover, various transition metal-chelating proteins such as albumin, ferritin and transferrin diminish or prevent the involvement of free iron or copper in radical reactions by complexing iron ions.[25]

The protecting role of small exogenous antioxidants, mostly derived from dietary fruits and vegetables,[31] has gained wide scientific interest. Examples of these small antioxidants are α-tocopherol (vitamin E), β-carotene and ascorbic acid. β-Carotene, which is a metabolic precursor of vitamin A, and α-tocopherol have been demonstrated to protect biological membranes from lipid peroxidation,[28] whereas ascorbic acid appears to act as a water-soluble radical scavenger in the cytoplasm. Hydrophobic and hydrophilic antioxidants can cooperate to reduce the antioxidant radicals that are formed. The vitamin E radical is, for instance, reduced by oxidation of GSH or vitamin C in the cytoplasm. Vitamin C, oxidized to dehydro-ascorbic acid, can in turn be reduced at the expense of GSH.

The defensive mechanisms as described above are not always sufficient to prevent the cell from damage by oxygen free radicals. Therefore, cells contain secondary antioxidant defense mechanisms, which involve DNA repair mechanisms, mechanisms of genomic surveillance, such as cell cycle checkpoints, and proteolytic degradation mechanisms.[25,32]

Oxidation and proteolytic degradation of proteins

Intracellular oxidative modification of proteins can occur through oxidation by free radicals or other activated species [33,34] or through oxidation catalyzed by mixed-function oxidases.[35,36] By any of these reactions, carbonyl groups, such as aldehydes and ketones, may be introduced into proteins.[37] As the thiol/SH group is very sensitive to oxidation, proteins containing cysteine (Cys) residues are excellent targets for redox-based modification.[38] Proteins can be directly oxidized, a process called primary modification. These modifications can be catalyzed by transition metals, such as Fe^{2+} and Cu^{+}, which bind to cation binding locations on proteins. Thereby, side chain amine groups on several amino acids are transformed into carbonyls with the aid of further attack by oxygen free radicals.[39] Reactions of proteins with molecules, generated by oxidation of other molecules, leads to secondary modification.[39]

Severe oxidized proteins undergo in many cases complete proteolytic degradation.[40] In eukaryotes, many proteins are degraded in a ubiquitin-dependent pathway.[41,42] In this pathway, numerous copies of an 8 kDa protein called ubiquitin are covalently linked to a target protein, which is subsequently degraded by a large ATP-dependent protease, called the 26S proteasome. Indeed, mild oxidative stress has been shown to enhance the ubiquitination of proteins and intracellular proteolysis, suggesting that the ubiquitin-dependent pathway is involved in removal of oxidatively damaged proteins.[43] However, it has also been demonstrated that severe oxidative stress results in a decrease of

the ubiquitination pathway.[44] Under these conditions, the cell might remove severe oxidized proteins by an ATP- and ubiquitin-independent proteolytic pathway that involves only the 20S core of the proteasome complex.[45,46]

Next to severe oxidative damage leading to degradation of proteins, more subtle changes occur that alter protein function such as enzymatic activity. The last decade, the effects of oxidative stress on protein-protein interactions involved in signal transduction, has gained much scientific interest. Signal transduction can elicit a wide range of cell-type specific responses leading to proliferation, migration, differentiation and apoptosis. It has become more and more apparent that changes in the cellular redox (SH) status can affect signal transduction pathways, gene expression and cellular responses such as cell proliferation,[29,47,48] indicating that oxidative stress might strongly affect cellular functioning. In this chapter, signal transduction will first be explained with the help of epidermal growth factor-induced signaling and subsequently, the effects of oxidative stress on signal transduction will be discussed.

Epidermal Growth Factor-induced Signal Transduction

Epidermal Growth Factor

Epidermal Growth Factor (EGF) was one of the first identified growth factors and was isolated from extracts of the male mouse submaxillary gland.[49] The mouse EGF has high homology with human EGF and urogastrone, a hormone that is isolated from human urine.[50-52] In humans, EGF is found in several body fluids such as urine, sweat, milk and cerebrospinal fluid and in a variety of tissues such as placenta, kidney, stomach, duodenum and bone marrow.[52-56] The protein itself is a nonglycosylated 53-amino acid polypeptide with a molecular weight of 6045 Dalton and is folded into a globular structure as the result of three intramolecular disulphide bonds. These disulphide bonds are formed by six conserved Cys residues, which are required for stabilization of the tertiary structure and for biological activity.[57,58] EGF is considered as one of the key regulators for epidermal growth and differentiation and is a potent mitogen for many cell types of ectodermal, mesodermal, and endodermal origin.[59]

The human EGF Receptor

EGF mediates its mitogenic responses in target cells by binding to the transmembrane EGF receptor that is expressed in a wide variety of cell types. The EGF receptor was the first receptor tyrosine kinase (RTK) to be purified and cloned [60] and is a member of the erbB family. The erbB family derives its name from the avian erythroblastosis retroviral oncogenes (v-erbB) and includes the mammalian EGF receptor/c-erbB1,

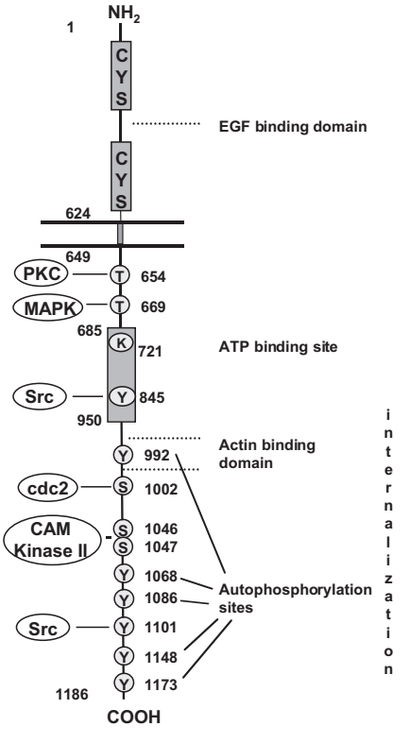

Fig. 3. Schematic representation of the EGF receptor (adapted from reference 61). The extracellular domain consists of two cysteine-rich regions (Cys) flanking the EGF binding domain. The transmembrane domain is located between amino acid residues 625 and 648. The intracellular part of the receptor contains several serine (S), threonine (T) and tyrosine (Y) phosphorylation sites, a tyrosine kinase domain (residues 685-950) containing the ATP binding site and an actin binding domain. Furthermore, the C-terminal region of the EGF receptor contains three internalization sequences as further described in the text.

HER2/neu/c-erbB2, HER3/c-erbB3 and HER4/c-erbB4, which differ in their ligand-binding and substrate specificity.[61] Increased expression and rearrangement of c-erbB receptors occur in various human tumors, suggesting an important role in human oncology.[62]

The human EGF receptor is a 170 kDa glycoprotein that consists of 1186 amino acid residues, organized in three main domains: the extra- and intracellular domains and a transmembrane domain. The extracellular domain, consisting of amino acid 1 to 624, is characterized by its capacity to bind EGF and EGF-related ligands with high affinity and can be subdivided into four domains. The N-terminal part of the EGF receptor (domain I) is heavily glycosylated on 12 potential sites and has a globular structure containing at least four short α-helices and β-sheets.[63-65] The EGF binding site (domain III) is localized between two Cys rich domains (domain II and IV) that form intrachain disulfide bridges that are important for maintenance of the tertiary structure of the receptor.[66,67] Binding of EGF to its receptor, which involves residues 351-364,[68] induces a conformational change of both the extracellular domain and the intracellular domain changing from a compact into a more elongated structure.[69,70] The transmembrane region of the EGF receptor, amino acid 625 to 648, is a short hydrophobic domain that spans the membrane as a single α-helix. The

intracellular domain, involving amino acid residues 649-1186, contains a tyrosine (Tyr) kinase domain from amino acid 685 to 950,[71] which is the most highly conserved domain of all RTKs. The Tyr kinase domain contains an ATP binding site at Lys-721 that is essential for receptor kinase activity.[72] Activation of the kinase domain results in the trans- or autophosphorylation of multiple Tyr residues in the C-terminal tail of the EGF receptor. Five sites of *in vivo* autophosphorylation have been identified: Tyr-992, 1068, 1086, 1148 and 1173.[73-75] Furthermore, the EGF receptor becomes phosphorylated on Tyr-845 and Tyr-1101 by the nonreceptor tyrosine kinase pp60c-Src,[76,77] and on serine (Ser) 1002, 1046 and 1047 and threonine (Thr) 654 and 669 by protein kinase C (PKC), mitogen-activated protein kinase (MAPK), calmodulin-dependent protein (CAM) kinase II and p34cdc2.[78-83] The intracellular domain of the EGF receptor also contains three sequences required for endocytosis, which involve residues 973-991, 993-1021 and 1023-1186.[84,85] These domains overlap the actin binding domain located from residues 984-996 [86] and contain a high affinity binding site for the clathrin adaptor protein complex AP-2 at Tyr-974.[87] Interaction with endocytic regions located in the regulatory C-terminus of the EGF receptor are proposed to be mediated by EGF-induced conformational changes that expose masked endocytic regions.[70] A schematic representation of the EGF receptor is shown in Figure 3.

Epidermal growth factor-induced receptor signaling

Binding of EGF to the extracellular domain of the receptor induces dimerization,[88,89] which involves the association of two monomeric EGF:EGF receptor complexes.[90] Stabilization of these complexes might be ligand-mediated or receptor-mediated, accomplished by ligand-induced conformational changes of the receptor.[91,92] Dimerization leads to stimulation of EGF receptor Tyr kinase activity which promotes the auto-phosphorylation of multiple C-terminal Tyr residues.[61] The function of this auto-phosphorylation is to generate high-affinity docking sites for downstream signaling molecules, which bind to the receptor via Src homology 2 (SH2)[93] or phosphotyrosine-binding (PTB) domains.[94] By auto-phosphorylation, the EGF receptor generates four docking sites, whereas heterologous phosphorylation, for example by pp60c-Src, can generate further sites.[76,77]. To date, at least 30 proteins have been identified to interact either directly or indirectly with the EGF receptor.[61] Proteins that interact directly with the receptor include phosphoinositide-specific phospholipase Cγ (PLCγ), ras GTPase activating protein (ras GAP), pp60c-Src,[95] Shc,[96] Grb2,[97] and the p85 kDa subunit of phosphatidylinositol 3-kinase (PI3-kinase).[98] Receptor binding of proteins with enzymatic activity results in Tyr phosphorylation and changes in their activity, whereas nonenzymatic proteins function as adaptor molecules and mediate the engagement of the activated EGF receptor with enzymatically active effector proteins. By interactions of these proteins, various signal transduction cascades are initiated and the extracellular signal is via the transmembrane

receptor transduced through the cytoplasm to the nucleus, which might finally lead to activation of nuclear transcription factors, altered gene expression and cell proliferation.

Mitogen-activated protein kinase signal transduction pathway

A family of kinases that plays a prominent role in the transduction of extracellular signals into intracellular events is formed by the mitogen-activated protein kinases (MAPKs).[99] The MAPK family can be divided into three subgroups: the stress-activated protein kinase/c-Jun N-terminal kinase, $p38^{MAPK}$ and the extracellular-regulated protein kinases (ERKs),[100-102] which all respond to different extracellular stimuli. Stimulation of cells with EGF results in the activation of $p44^{MAPK}$ and $p42^{MAPK}$ (ERK1 and ERK2, respectively). Upon EGF-induced auto-phosphorylation of the EGF receptor a pre-existing Grb2-Sos complex binds to the receptor (Fig. 4). This complex binds either directly to Tyr-1068 of the EGF receptor via the SH2 domain of Grb2,[103] or indirectly through binding to Shc, which is bound to the EGF receptor via its PTB domain.[96] Sos then activates Ras by catalyzing the GDP/GTP exchange[104] and this leads to the activation and recruitment of the Ser/Thr kinase Raf-1 to the plasma membrane. Negative feedback on Ras is regulated by the GTPase activating protein GAP.[105] Once activated, Raf-1 is released from the plasma membrane and

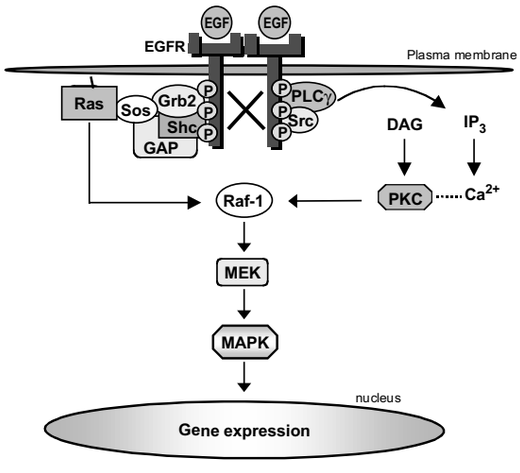

Fig. 4. EGF-induced signal transduction pathway leading to the activation of MAP kinase (MAPK). Stimulation of cells with EGF induces dimerization and subsequent cross- or autophosphorylation of the EGF receptor (EGFR). The Tyr-phosphorylated sites then function as docking sites for downstream signaling molecules that are recruited to the EGFR and become activated. Via protein-protein interactions the cytosolic Ser/Thr kinase Raf-1 becomes activated, leading to the phosphorylation and activation of MEK and MAPK. MAPK can subsequently enter the nucleus where it can activate transcription factors, leading to altered gene expression and a cellular response.

subsequently activates MAPK kinase MEK that, in turn, catalyzes a dual phosphorylation of p44/p42^{MAPK} on Thr and Tyr residues.[106,107] Activation of Raf-1 can also be accomplished by EGF-induced activation of PLCγ, which induces hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce inositol-1,4,5-trisphophate (IP_3) and diacylglycerol (DAG). IP_3 subsequently binds to receptors on intracellular Ca^{2+} stores and triggers a Ca^{2+} response. DAG and Ca^{2+} then activate the Ser/Thr kinase PKC, which subsequently activates Raf-1 by direct phosphorylation.[108] Activated p44/p42^{MAPK} can phosphorylate target proteins in the cytoplasm, such as phospholipase A2,[109] or translocate to the nucleus where it can activate transcription factors, such as c-myc and c-jun,[110] finally leading to altered gene expression and a cellular response (Fig. 4).

Regulation of EGF receptor signaling

Protein tyrosine phosphatases

Besides the activation of Tyr kinases, EGF also induces the activation of protein-tyrosine phosphatases (PTPs), which can negatively regulate growth factor signaling. Dephosphorylation of the C-terminal Tyr residues of the EGF receptor, for instance, abrogates docking sites for downstream signaling proteins and in addition, enzymatic activity of signaling proteins can be negatively regulated by dephosphorylation. The PTP superfamily is divided in three groups: I) the low molecular weight PTPs (LMW-PTPs), II) the dual specificity phosphatases (DSPs) and III) the classical protein-tyrosine phosphatases (PTPs). Although LMW-PTPs share little sequence conservation with other PTPs, they have the same catalytic mechanism with a central catalytic Cys. LMW-PTPs have been involved in platelet-derived growth factor (PDGF) receptor signaling and furthermore, LMW-PTPs may be regulated by Src-dependent Tyr phosphorylation and H_2O_2.[111,112] DSPs dephosphorylate *in vitro* phospho (p) Ser, pThr and pTyr but have *in vivo* preference for pTyr and pThr. The DSP family consists of evolutionary conserved members, including the MAPK phosphatase MKP-1,[113] and the cdc25 cell cycle phosphatases.[114] Classical PTPs are proteins containing a PTP domain of 250-300 amino acids that contains the essential catalytic site Cys. PTP activity is specific for pTyr residues, due to the depth of the active-site cleft in which only pTyr is long enough to reach the catalytic Cys.

The classical PTP group is further subdivided into two families, based on their cellular compartmentalization, in receptor PTPs (RPTPs) and intracellular PTPs. RPTPs consist of a variable extracellular domain, followed by a single transmembrane region and an intracellular domain. The intracellular region of most RPTPs contains two PTP domains of which the membrane proximal domain retains most if not all activity.[115] RPTPα is a typical RPTP that becomes Tyr phosphorylated and binds to Grb2.[116] Recently, phosphorylated RPTPα was shown to activate pp60c-Src by dephosphorylating pTyr527,[117] indicating that

phosphatases can also positively regulate cell signaling. The intracellular PTPs have a single PTP domain and a great diversity of additional protein domains that regulate catalytic activity, the targeting of PTPs to specific subcellular locations and the binding to specific substrates. For example, cleavage of the hydrophobic C-terminal region of PTP1B, that directs PTP1B to the endoplasmic reticulum, results in its release into the cytoplasm.[118] Other members of this group of PTPs are PTP1C (also called SHP-1) and PTP1D (also called SHP-2 or Syp). PTP1C has been shown to interact with the EGF receptor and is involved in EGF receptor dephosphorylation.[119] PTP1D also associates with the EGF receptor and becomes Tyr phosphorylated and thereby activated upon EGF stimulation.[120] By the activation of PTPs, the dephosphorylation of the EGF receptor and other Tyr phosphorylated proteins is initiated, resulting in attenuation of EGF-induced signal transduction.

EGF receptor transmodulation

Another rapid mechanism to regulate EGF-induced EGF receptor signaling is to lower the affinity of the receptor for its ligand and to inhibit receptor Tyr kinase activity. This process is referred to as desensitization or transmodulation and is mainly accomplished by phosphorylation of the receptor on Thr and Ser residues. Phosphorylation of the EGF receptor on Thr-654 by PKC results in reduced Tyr kinase activity and, although Thr-654 is not essential, its phosphorylation by PKC can induce a decrease in receptor high affinity, a process in which MAPK kinase has been involved.[121-124] Moreover, phosphorylation of Thr-669 by MAPK and phosphorylation of Ser-1046 and Ser-1047 by CAM kinase II might be involved in receptor desensitization as well.[79,81]

EGF receptor downregulation

The third mechanism of negative feedback upon EGF stimulation is referred to as receptor downregulation. This includes the internalization and subsequent degradation of activated EGF receptors and results in a loss of EGF binding sites at the plasma membrane. In non-stimulated cells, the EGF receptor is internalized by a relatively slow basal pathway in which it cycles between the plasma membrane and endosomes. Phosphorylation of the EGF receptor at Thr-654 by PKC has been proposed to be involved in both the internalization and recycling of nonoccupied receptors.[125] Upon EGF-induced activation, EGF receptors are recruited to specialized regions in the plasma membrane, the clathrin-coated pits (Fig. 5).[126] Clathrin is attached to the membrane via the adaptor protein AP-2, which is a major component of coated pits.[127] Furthermore, AP-2 interacts with the EGF receptor, mediated by the receptor C-terminal internalization sequence containing Tyr-974.[87] Another protein that is recruited to the plasma membrane upon EGF stimulation is the AP-2 binding protein EGF receptor pathway substrate clone 15 (Eps15).[128-130] Upon recruitment to the plasma membrane, Eps15 is located at the rim of coated pits[131] and it was recently demonstrated

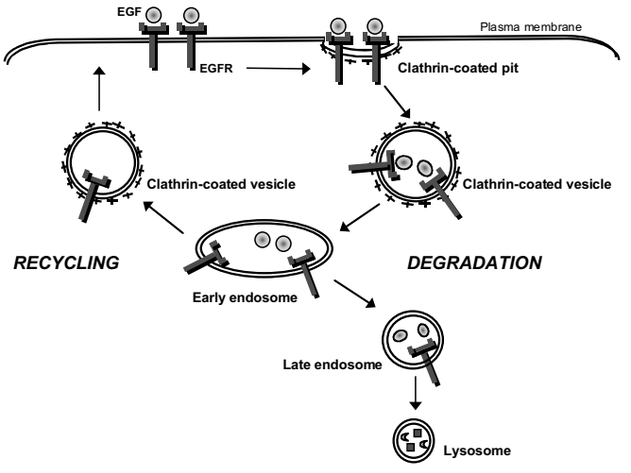

Fig. 5. EGF-induced endocytosis of the EGF receptor. Upon EGF-induced activation, the EGFRs are recruited towards the clathrin-coated pit that buds off from the plasma membrane, thereby forming a clathrin-coated vesicle. After uncoating, the vesicle fuses with the early endosome where sorting takes place. The receptor can, via clathrin-coated vesicles, be recycled to the plasma membrane. However, the majority of the EGFR will be transported to the late endosomes and finally to the lysosomes where they become degraded.

that Tyr phosphorylation of Eps15 is required for ligand-induced internalization of the EGF receptor.[132] After clustering of EGF receptors[133] in coated pits, these pits bud of from the plasma membrane to form clathrin-coated vesicles. The pinching off of vesicles is dependent on the GTPase dynamin,[134] which has been shown to bind to PLCγ, Grb2 and PIP_2.[135,136] PIP_2 stimulates GTP hydrolysis by dynamin, but hydrolysis is stimulated even further when Grb2 is present.[136] After uncoating, the vesicles fuse with early endosomes, where sorting takes place. Although many endocytosed integral membrane proteins recycle, the majority of EGF receptors are targeted to late endosomes and lysosomes for degradation.[59,137-139] Sorting of the internalized receptor is regulated, in part, by the intrinsic Tyr kinase activity of the receptor.[138] However, it has become evident that ubiquitination of the EGF receptor might also target receptors to the late endosome, a compartment where both proteasomal and lysosomal hydrolyses may respectively degrade the cytoplasmic and exoplasmic domains.[140,141]

Although it was generally thought that internalization of active receptors occurs only to attenuate the EGF-induced response, it is now becoming more and more apparent that receptor signaling and receptor endocytosis are two inseparable pathways that work together in both positive and negative regulation of receptor activity. Because of the cytosolic

orientation of the Tyr-phosphorylated tail and the presence of an active EGF receptor Tyr kinase in endosomes it is suggested that the receptor may continue to signal after internalization.[142-144] Indeed, a population of Shc proteins becomes phosphorylated by EGF receptors located at the endosome, hereby serving as an effective amplification mechanism for accessing Ras.[145] Moreover, isolation of endosomes from hepatocytes showed that the early-endosome compartment contained active Raf-1 and MEK, suggesting that signal transduction is maintained after internalization of the EGF receptor.[146]

Oxidative stress and signal transduction

Activation of signaling proteins by oxygen free radicals

Although the biological production of oxygen free radicals has been known for a long time, the effects of oxidative stress on signal transduction and cellular functioning have been studied widely for the last decade. Oxygen free radicals have been shown to have diverse effects on cell functioning, probably depending on both the dose and duration of exposure. The involvement of free radicals in the process of cancer suggests that oxidative stress is capable of inducing cell proliferation, whereas aging, in which oxidative stress is involved as well, is accompanied by growth arrest and even programmed cell death. Since these processes are, at least partly, mediated and controlled by cascades of phosphorylations and dephosphorylations of cytoplasmic proteins, this suggests that oxidants affect intrinsic signal transduction pathways.

Various studies have indeed established that oxygen free radicals can cause the phosphorylation and activation of numerous signaling proteins, including RTKs,[147,148] PKC,[149] PLCγ1,[150] Src kinases,[151,152] MAPKs,[153,154] protein kinase B,[155] and transcription factors.[156,157] As an example, only the effects on the MAPK pathway will be discussed. Exposure of cells to UV light, $ONOO^-$ or H_2O_2 induces phosphorylation of the PDGF receptor and the EGF receptor,[147,148,158-162] suggesting that oxygen free radicals initiate signaling events that mimic those induced by growth factors. This was confirmed by the observation that the H_2O_2-induced Tyr phosphorylated EGF receptor forms a complex with Shc, Grb2 and Sos in vascular smooth muscle cells, followed by the activation of $p21^{ras}$.[163] Furthermore, oxidative stress-induced phosphorylation and activation of the Ser/Thr kinase Raf-1, a downstream effector molecule of $p21^{ras}$, has been described in several cell types as well.[164,165] Since only the membrane-associated form of Raf-1 has been proposed to become Tyr-phosphorylated,[166-168] this suggests that ROS cause the recruitment of Raf-1 to the plasma membrane, followed by its Tyr phosphorylation.

Other studies have established that oxidative stress induces the phosphorylation and activation of MAPKs.[153,154,162,169-171] Activation of $p42/p44^{MAPK}$ by ROS might be due to direct phosphorylation of MEK, for instance by exposure of cells to $ONOO^-$.[162] On the other

hand, activation of p42/p44^{MAPK} by H_2O_2 has been shown to be mediated by the activation of the RTK, PKC, Raf-1 and MEK,[165] indicating that different ROS might have different targets. Furthermore, activation of MAPKs by oxygen free radicals has been shown to result in subsequent activation of transcription factors and induction of c-fos and c-jun expression.[172-176] The oxidant-induced MAPK activation might be involved in the cellular response to oxidative stress.[169,177] Both dose and duration of exposure probably determine whether activation of MAPKs by ROS will finally result in cell survival or in cell death.

ROS as second messengers in signal transduction and their potential targets

The effects of oxygen free radicals on signal transduction might be derived from normal cell physiology. A variety of ligands, including tumor necrosis factor-α,[178] interleukin-1,[178] transforming growth factor-β1,[179] PDGF,[180] and EGF,[181] have been demonstrated to produce ROS upon receptor binding. The generation of oxidants by ligand-receptor interactions resulted in stimulation of signaling molecules and therefore, ROS are nowadays considered to serve as physiologic second messengers in signal transduction. The predominant ROS produced upon EGF and PDGF stimulation appeared to be H_2O_2 and furthermore, EGF receptor activation induced by UV radiation is also mediated by H_2O_2.[180-182] Elimination of H_2O_2 by incorporation of catalase revealed to inhibit EGF-induced Tyr phosphorylation of various signaling proteins including the EGF receptor.[181] In addition, it was shown that EGF-induced H_2O_2 production required intrinsic RTK activity, but probably not the C-terminal autophosphorylation sites of the EGF receptor.[181] These studies suggest that activation of a RTK upon binding of a growth factor may not be sufficient to increase the steady state level of protein Tyr phosphorylation. Therefore, the cell might achieve an increase in Tyr phosphorylation by the concomitant production of H_2O_2, as will be discussed below.

An interesting question is how ROS do accomplish the phosphorylation and activation of signal transduction component molecules. Both the activation of Tyr kinases and the inactivation of Tyr phosphatases have been proposed. In the last few years, several studies have shown that exposure of cells to H_2O_2, UV radiation or $O_2^{\cdot-}$ causes the (reversible) inactivation of different PTPs, including LMW-PTPs, RPTPα, PTP1B and PTP1C.[183-186] Furthermore, stimulation of A431 cells with EGF resulted in a reversible inactivation of PTP1B.[187] The PTPs that are attacked by ROS all seem to share the same catalytic mechanism with a central catalytic Cys. Since SH groups are fairly sensitive to oxidation, increased phosphorylation of signaling proteins by ROS are most likely accomplished by reversible inactivation of PTPs via oxidation of essential SH groups within their active site Cys,[187] thereby assuming that the cell maintains spontaneous Tyr kinase activity.[160] Therefore, these studies suggest that ROS, either generated intracellularly in response to EGF or added extracellularly, act as second messengers in signal transduction and activate signaling pathways by the same mechanisms.

Scope of this thesis

Oxygen free radicals have diverse effects on proteins involved in signal transduction, probably mediated via reversible inactivation of PTP. This thesis focuses on the effects of oxidative stress on receptor downregulation, which is another cellular negative feedback mechanism to attenuate growth factor-induced cell signaling. Furthermore, it was the aim of this thesis to use oxidant-induced modifications on protein levels as tools to develop large scale screening assays in cellular systems that could subsequently be used to screen the efficacy of large numbers of antioxidants to protect the cell against oxidative damage. **Chapter 2** describes the development of a cellular large scale-screening assay to measure the phosphorylation of p44/p42^{MAPK}. In this chapter, the phosphorylation of p44/p42^{MAPK} is used as a marker for oxidative stress and with the help of phosphospecific antibodies a cellular enzyme-linked immunosorbent assay (Cell-ELISA) in 96-well plates was developed. In addition, some results of the application of this newly developed screening method are described. **Chapter 3** shows the effect of H_2O_2 on EGF receptor-mediated endocytosis in fibroblast cells. The results show that H_2O_2 inhibits the internalization of the EGF receptor in a concentration-dependent manner. Furthermore, the EGF-induced mono-ubiquitination of Eps15 was found inhibited in the presence of H_2O_2. Based on these findings it is suggested that H_2O_2 inhibits EGF receptor internalization by an inhibition of ubiquitination of proteins involved in the internalization process. In **chapter 4** the development of a nonradioactive large scale-screening assay to measure EGF receptor internalization in 96-well plates is described. This assay is partly based on the assay as described in *chapter 2* and it is concluded that the newly developed assay is a reliable tool to use for the screening of compounds that interfere with EGF receptor internalization. **Chapter 5** deals with the mechanism underlying the oxidant-induced inhibition of EGF receptor-mediated endocytosis as described in *chapter 3*. The internalization of the EGF receptor and ubiquitination of both Eps15 and the EGF receptor were reversibly inhibited by H_2O_2. In addition, the concentration-dependent inhibition of EGF receptor internalization correlated with a concentration-dependent increase in the cellular ratio of GSSG:GSH and furthermore, increased GSSG:GSH ratios recovered to control levels upon H_2O_2-removal prior to re-establishment of ubiquitination and EGF receptor internalization. Therefore, it is concluded that the results shown in this chapter strengthen the hypothesis that H_2O_2 inhibits EGF receptor internalization by an inhibition of ubiquitination of proteins involved in the internalization process as previously described in *chapter 3*. Finally, the mechanisms underlying H_2O_2-induced inhibition of EGF receptor internalization and the possible consequences for cellular functioning are further discussed in **chapter 6**.

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Chapter 2

Large Scale Screening Assay for the Phosphorylation of Mitogen-Activated Protein Kinase in Cells

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Abstract

Mitogen-activated protein (MAP) kinases are serine/threonine kinases that are activated by phosphorylation and are involved in the cellular response to various physiologic stimuli and stress conditions. Because MAP kinases play an important role in cellular functioning, a screening assay to determine the phosphorylation of MAP kinase upon various conditions was desirable. Therefore, we have developed a cellular enzyme-linked immunosorbent assay (Cell-ELISA), in which the phosphorylated forms of p42^{MAPK} and p44^{MAPK} are detected. We show that in this Cell-ELISA, MAP kinase becomes phosphorylated in a dose- and time-dependent manner under proliferative or stress conditions. This dose- and time-dependent phosphorylation agrees with observations using classical gel-electrophoresis and Western blotting techniques. Furthermore, we show that our assay is applicable to different cell types and that serum-starvation is not required for detection of an increase in MAP kinase phosphorylation. From these experiments, it is concluded that the Cell-ELISA is a reliable and fast method for quantitative detection of the phosphorylation, and thus the activation, of MAP kinase. This assay is applicable for a large-scale screening of the effectivity of biological or chemical compounds that modulate the cellular response to physiologic stimuli or stress through phosphorylation and activation of MAP kinase.

Introduction

A family of kinases that plays a prominent role in the transduction of extracellular signals into intracellular events is formed by the mitogen-activated protein (MAP) kinases.[1,2] MAP kinases are serine/threonine protein kinases that are rapidly activated in response to various extracellular signals, such as growth factors, cytokines and different types of cellular stress.[3-8] Prolonged activation of these kinases is required for cellular responses such as proliferation and differentiation.[9-13] Furthermore, MAP kinases have been implicated in cell survival following sublethal levels of oxidative stress or, in contrast, in cell death under severe stress conditions.[6,14]

The family of the MAP kinases can be divided into three subgroups: the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38 MAP kinase (p38^{MAPK}) and the extracellular-regulated protein kinases (ERKs).[15-18] Activation of MAP kinases occurs through a dual phosphorylation on Thr and Tyr residues, catalyzed by MAP kinase kinases.[19-21] For the p42^{MAPK}/ERK2 isoform, phosphorylation of Thr is supposed to provide a correct alignment of catalytic residues, whereas phosphorylation of Tyr might facilitate the binding of protein substrates.[22] Deactivation of MAP kinases is accomplished by protein phosphatases that dephosphorylate either the regulatory Thr or the Tyr residue, or both.[23]

Because phosphorylation of MAP kinase leads to its activation, phosphorylation of MAP kinase reflects its activity. As mentioned, the stress-induced phosphorylation of MAP kinase occurs rapidly and therefore, this event might be used as a marker for cellular stress. Considering this, it would be of interest to develop an assay in which the phosphorylation of MAP kinase is used as a tool for screening the effectivity of compounds and conditions that induce or, in contrast, reduce cellular stress. In this study, we have developed a cellular enzyme-linked immunosorbent assay (Cell-ELISA) for the phosphorylation of the $p44^{MAPK}$ and $p42^{MAPK}$ isoforms (ERK1 and ERK2, respectively). We show that in our assay, treatment of cells with H_2O_2, which is an inducer of oxidative stress, leads to a time- and dose-dependent phosphorylation of MAP kinase, according to data obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assays and kinase activity assays.[6] Furthermore, we show that the Cell-ELISA is not restricted to one cell type, and that a transient phosphorylation of MAP kinase is accomplished under both proliferative and stress conditions. Therefore, it is concluded that the Cell-ELISA is a reliable screening method for the phosphorylation of MAP kinase that could be used for screening the effectivity of biological or chemical compounds that modulate cellular stress.

Materials and Methods

Cell culture

Rat-1 fibroblasts were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% fetal calf serum (Gibco) in a 7.5% CO_2 humidified atmosphere. Neonatal myocardial cells were cultured according to the method of Harary and Farley as described previously.[24,25] Myocardial cells were plated on Primaria 96-wells plates (Falcon®, Becton Dickinson Labware, Oxford, UK) in growth medium [(Gibco), Ham F10, supplemented with 10% fetal calf serum (Gibco), penicillin (100 U/ml)/streptomycin (0.1 mg/ml), 10 µM cytosine ß-D-arabino furanoside (Sigma, St. Louis, MO) and 1 mM $CaCl_2$] in a 7.5% CO_2 humidified atmosphere at 37°C. Before treatment with platelet-derived growth factor (PDGF) or H_2O_2, Rat-1 cells were serum-starved in DMEM for at least 15 hr. Because serum might be required as a carrier for lipophilic compounds to be tested in the Cell-ELISA, we also performed the Cell-ELISA in cells without serum-starvation.

Antibodies

The monoclonal antibody directed against $p42^{MAPK}$ was obtained from UBI Upstate Biotechnology, Lake Placid, NY; the rabbit antibody directed against phosphorylated $p44/42^{MAPK}$ was purchased from New England Biolabs Inc., Beverly, MA. Horseradish peroxidase-conjugated secondary goat anti-mouse (GAM-PO) and goat anti-rabbit (GAR-PO) antibodies and biotinylated goat anti-mouse (GAM-Bio) and goat anti-rabbit (GAR-Bio) antibodies were from Jackson ImmunoResearch Laboratories Inc., West Grove, PA.

MAP kinase phosphorylation in six-well plates

For MAP kinase phosphorylation assays, Rat-1 cells were grown on six-well plates (Nunc, Life Technologies, Breda, the Netherlands) to a confluency of 30,000 cells/cm^2. After serum-starvation, cells were washed once with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.2) supplemented with 5 mM glucose (PBS_{gluc}) before treatment with PDGF (20 ng/ml) or with 100 µM H_2O_2 in PBS_{gluc} at 37°C for the indicated times. After treatment, cells were washed once with ice-cold PBS, lysed in sample buffer (8.3% glycerol, 75 mM dithiothreitol, 1.7% sodium dodecylsulfate, 0.0025% bromophenol blue and 20 mM Tris-HCl pH 6.8) and boiled for 10 min.

SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE on a 12.6% polyacrylamide gel with an acrylamide/bisacrylamide ratio of 167:1 and subsequently transferred to PVDF membrane (Boehringer Mannheim, Germany). Blots were blocked for 1 hr at room temperature with 2% milk powder (Protifar, Nutricia, Zoetermeer, the Netherlands) in PBS without 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-0) with 0.05% (v/v) Tween-20 (PBST), followed by incubation with primary antibodies diluted in 0.5% milk powder in PBST for 1 hr at room temperature. After washing, blots were incubated for 1 hr at room temperature with secondary horseradish peroxidase-coupled antibodies diluted in the same buffer as used for the primary antibodies. Proteins were detected using the chemiluminescene procedure as described by the manufacturer (Life Science Products, Boston, MA).

Cell-ELISA

For the ELISA, Rat-1 fibroblasts were grown on 96-well plates (Nunc) to a confluency of 90,000 cells/cm^2. After washing with PBS_{gluc}, cells were treated with PDGF (20 ng/ml) or with H_2O_2 in PBS_{gluc} at 37°C for the indicated times. Subsequently, cells were fixed and permeabilized with 3.5% paraformaldehyde, 0.25% glutaraldehyde, 0.25% Triton X-100 in PBS-0 for 30 min at 37°C. Next, cells were washed once with PBS-0, treated two times for 5 min with 50 mM glycin in PBS-0, and blocked with PBS-0 containing 2% gelatin and 0.05% (v/v) Tween-20 for 30 min at 37°C. After washing once with 0.2% gelatin in PBS-0, different wells were incubated with the first antibodies (anti-$p42^{MAPK}$ 1:2000; anti-phospho-p44/$p42^{MAPK}$ 1:2000) diluted in 0.2% gelatin in PBS-0 for 1 hr at 37°C, followed by washing in the same buffer for 30 min at 37°C. Then, cells were incubated with the different biotinylated secondary antibodies (GAM-Bio 1:4000; GAR-Bio 1:4000) diluted in the same buffer as used for the primary antibodies for 1 hr at 37°C. After washing for 30 min at 37°C, cells were exposed to horseradish peroxidase-conjugated streptavidin (streptavidin-PO) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:20,000 in 0.2% gelatin in PBS-0 for 45 min at 37°C. Extensive washing for 30 min at 37 °C was followed by incubation with the horseradish peroxidase substrate *o*-Phenylenediamine Dihydrochloride (OPD) (3.7 mM) in 50 mM Na_2HPO_4 and 25 mM $C_6H_8O_7{\cdot}H_2O$ for 25 min in the dark at

room temperature. The reaction was stopped by the addition of 50 vol% 1 M H_2SO_4 and spectrophotometric readings were performed at 490 nm using a Microplate Reader (Benchmark, Bio-Rad Laboratories, Inc., Hercules, CA). Using the software belonging to the microplate reader, data can be exported into a spreadsheet for subsequent analysis.

In each experiment, total amounts of $p42^{MAPK}$ were used as a control of equal amounts of protein and cells in each well. Incubations with the secondary biotinylated antibodies and the streptavidin-PO were used to test for any effect of the different treatments on aspecific labeling of the antibodies. For determination of significant changes after treatment of cells, statistical analysis of the data was performed according to the Student's *t* test.

In order to investigate whether the developed Cell-ELISA is also applicable to other cell types, a series of experiments were performed using a primary culture of neonatal rat heart myocytes. The cells were plated in 96-well plates (Falcon) with a density of 100.000 cells/cm^2. The cells were used 6 days upon isolation, in which time a monolayer of spontaneously beating cells was formed. The cells were incubated with 50 µM H_2O_2 and processed as described above.

Results and Discussion

PDGF and H_2O_2 induce phosphorylation of MAP Kinase

Considering that phosphorylation of MAP kinase leads to its activation, we developed a screening assay in which phosphorylation of MAP kinase was regarded as a measure of its activity. Initially, phosphorylation of $p42^{MAPK}$ was determined by SDS-PAGE. After its phosphorylation, the mobility of $p42^{MAPK}$ is changed and therefore, the phosphorylated form of $p42^{MAPK}$ can be distinguished from the non-phosphorylated form on Western blot.[26] Figure 1 shows that both PDGF and H_2O_2, which is an inducer of oxidative stress, stimulated a shift in the electrophoretic mobility of the $p42^{MAPK}$ protein in Rat-1 cells. This indicates that $p42^{MAPK}$ is phosphorylated under both proliferative and stress conditions.

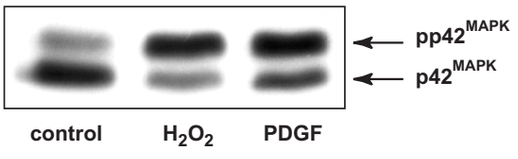

Fig. 1. Phosphorylation of $p42^{MAPK}$ by H_2O_2 and PDGF. Rat-1 fibroblasts were treated with 100 µM H_2O_2 or with PDGF (20 ng/ml) for 20 min at 37°C. Subsequently cells were lysed in sample buffer and proteins were separated by 12.6% SDS-PAGE, transferred to PVDF membrane and immunodetected with monoclonal anti-$p42^{MAPK}$ antibody as described in *Materials and Methods*. $p42^{MAPK}$: non-phosphorylated form of $p42^{MAPK}$; $pp42^{MAPK}$: phosphorylated form of $p42^{MAPK}$.

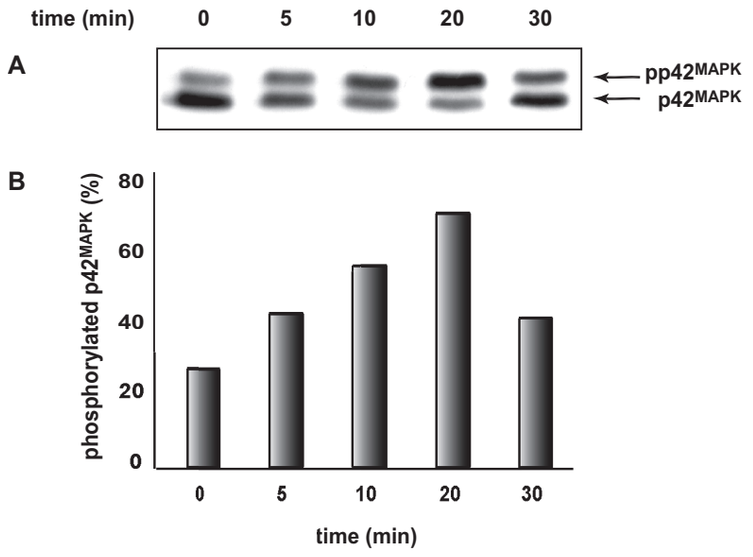

Fig. 2. Time course for phosphorylation of $p42^{MAPK}$ by H_2O_2. Rat-1 fibroblasts were treated with 100 µM H_2O_2 for the indicated times, after which cells were harvested and loaded on a 12.6% SDS-polyacrylamide gel. (A) Detection of $p42^{MAPK}$ with monoclonal anti-$p42^{MAPK}$ antibody on Western blot. $p42^{MAPK}$: non-phosphorylated form of $p42^{MAPK}$; $pp42^{MAPK}$: phosphorylated form of $p42^{MAPK}$. (B) Relative amount of phosphorylated $p42^{MAPK}$. Above bands were scanned by densitometry and quantified using the ImageQuant software. The amounts of phosphorylated $p42^{MAPK}$ compared to the total amount of $p42^{MAPK}$ are depicted as arbitrary units.

The kinetics of $p42^{MAPK}$ phosphorylation induced by H_2O_2 (Fig. 2) show that phosphorylation occurred within 5 min of treatment and declined after 20 min. This transient phosphorylation is in agreement with data published previously, which show that the activation of $p42^{MAPK}$ is time-dependent after treatment of cells with H_2O_2 or growth factor.[6,7,27,28]

The above described analysis of the electrophoretic mobility shift involves several steps that both involve long periods of time and have only limited capacity, and therefore, this assay is not efficient for large-scale screening. Therefore, we developed a Cell-ELISA in 96-well plates, in which an antibody that only recognizes the phosphorylated forms of both $p42^{MAPK}$ and $p44^{MAPK}$ was used. With this antibody, the phosphorylated forms of MAP kinase can be specifically detected in a mixture of proteins or even within cells without further processing of the protein samples. First, we tested this polyclonal antibody by SDS-PAGE. Detection of $p42^{MAPK}$ and $p44^{MAPK}$ with the phospho-specific antibody on Western blot after treatment of Rat-1 fibroblasts with H_2O_2 for the indicated times showed a transient phosphorylation of both MAP kinases (Fig. 3). Detection of the same blot with anti-$p42^{MAPK}$

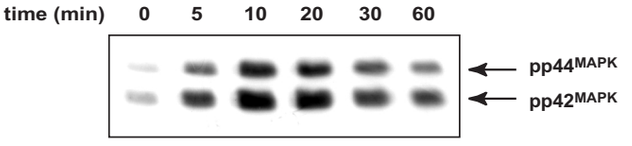

Fig. 3. Transient phosphorylation of p42^{MAPK} and p44^{MAPK}. Rat-1 fibroblasts were treated with 100 µM H_2O_2 for the indicated times. After harvesting of the cells, proteins were separated by 12.6% SDS-PAGE and the phosphorylated forms of p42^{MAPK} (pp42^{MAPK}) and p44^{MAPK} (pp44^{MAPK}) were detected with polyclonal phospho-specific p44/42^{MAPK} antibody on Western blot.

antibody revealed that the total amount of the p42^{MAPK} protein remained unchanged (not shown). This indicates that the decrease in phosphorylation of MAP kinase after treatment for 20 min was not due to cell lysis or degradation of the protein, but was probably caused by phosphatase activity. Because the time-dependent phosphorylation shown in Figure 3 is in agreement with the pattern obtained by the electrophoretic mobility shift assays (Fig. 2), the phospho-specific p44/p42^{MAPK} antibody is a reliable tool to use for the development of a Cell-ELISA.

MAP kinase phosphorylation in 96-well plates

The availability of the antibody that only recognizes the phosphorylated form of p44/p42^{MAPK} provided the ability to develop an ELISA-based assay in cells. Therefore, Rat-1 fibroblasts were cultured in 96-well plates and were treated with H_2O_2 for the indicated time. After fixation and permeabilization of the cells, the phosphorylated forms of MAP kinase were determined with the polyclonal phospho-specific p44/p42^{MAPK} antibody in both control and H_2O_2-treated cells. As a control for H_2O_2-induced cell lysis or for degradation of the MAP kinase protein, the total amount of p42^{MAPK} was detected with the monoclonal anti-p42^{MAPK} antibody in different wells on the same plate as described in *Materials and Methods*. In each experiment, background values were obtained from cells treated in an identical manner, except for the incubations with the primary antibody. In the Cell-ELISA, the biotinylated secondary antibodies were used for amplification of the signal, thereby increasing the sensitivity of the detection. Subsequent incubation of the cells with streptavidin-PO was followed by the addition of OPD. After the reaction was stopped, spectrophotometric readings were performed as described in *Materials and Methods*.

As shown in Figure 4, treatment of Rat-1 fibroblasts with 50 µM H_2O_2 for 20 min induced a significant increase in the phosphorylation of MAP kinase as shown by the increased absorbance at 490 nm. Based on the data obtained from Western blotting (Fig. 3),

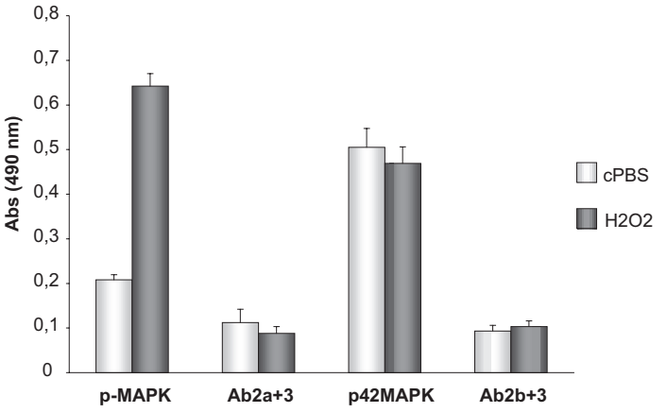

Fig. 4. Detection of MAP kinases in 96-well plates. Rat-1 fibroblasts were grown in 96-well plates and treated with 50 µM H_2O_2 for 20 min. The phosphorylated forms of MAP kinase (p-MAPK) were detected with a polyclonal phospho-specific p44/p42^{MAPK} antibody, whereas the total amount of p42^{MAPK} was determined using a monoclonal anti-p42^{MAPK} antibody as described in *Materials and Methods*. For background values, incubations with the primary antibodies were omitted. Ab2a+3: incubation with biotinylated goat anti-rabbit antibody and streptavidin-PO; Ab2b+3: incubation with biotinylated goat anti-mouse antibody and streptavidin-PO. Detections with different antibodies were performed in different wells. Results ± SD of a representative experiment are shown (*n*=8).

it must be concluded that this increase was induced by the phosphorylation of both p42^{MAPK} and p44^{MAPK}. Figure 4 also shows that the total amount of p42^{MAPK} was not influenced by treatment with H_2O_2 ($p > 0.05$). This again indicates that treatment with H_2O_2 for 20 min does not induce protein degradation or cell lysis. The latter is in agreement with data obtained in other experiments in which lactate dehydrogenase (LDH) release was negligible after treatment of cells with same amounts of H_2O_2 (not shown). Statistical analysis revealed that background values of the secondary biotinylated goat anti-rabbit and goat anti-mouse antibodies and of the streptavidin-PO were not significantly changed after treatment of Rat-1 cells with H_2O_2 (Fig. 4), showing that H_2O_2 does not influence the aspecific binding of the antibodies. Because the total amount of p42^{MAPK} and background values remained unchanged after treatment of cells, it is not necessary to correct for these values for determination of the increase in phosphorylation of MAP kinase. This increase in phosphorylation was determined by calculation of the following ratio:

$$\frac{Y \pm SD}{X \pm SD}$$

where Y is the phosphorylated p44/p42^{MAPK} after treatment of cells, X is the phosphorylated p44/p42^{MAPK} in untreated cells and SD is the standard deviation.

Calculation of this ratio with the data from the experiment shown in Figure 4 revealed that in this experiment, treatment with H_2O_2 gave a threefold increase in the phosphorylation of MAP kinase as compared with control cells.

H_2O_2 and PDGF induce a transient phosphorylation of MAP kinase in the Cell-ELISA

On Western blot we showed that the phosphorylation of MAP kinase induced by H_2O_2 was time-dependent with an optimum at 20 min (Fig. 2). To determine the validity of the Cell-ELISA by determination of a transient phosphorylation of MAP kinase, time-course experiments with both PDGF and H_2O_2 were performed. Total amounts of p42^{MAPK} and background values of the biotinylated secondary antibodies and the streptavidin-PO were again not affected by different treatments of the cells (data not shown). The relative increases in phosphorylation of MAP kinase after treatment of Rat-1 cells compared with untreated cells were determined as described above. Figure 5 shows that both H_2O_2 and PDGF induced a transient phosphorylation of MAP kinase with an optimum at 20 min, which is in agreement with the data obtained by Western blotting (Fig. 2) and with previously published data.[7,27] This indicates that the transient phosphorylation of MAP kinase is not dependent on experimental conditions and that the Cell-ELISA is a reliable method to quantitatively determine the degree of phosphorylation of MAP kinase.

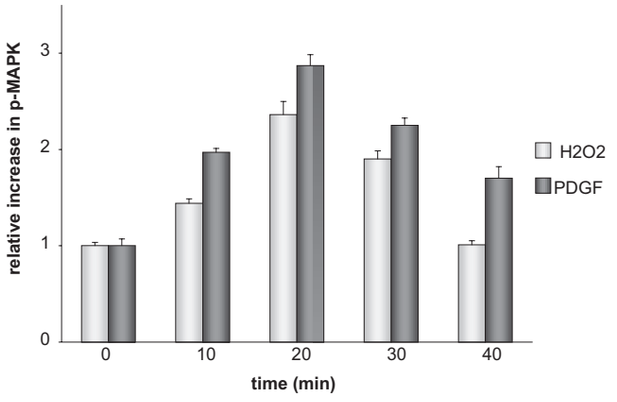

Fig. 5. Time-dependent increase in the phosphorylation of MAP kinase induced by PDGF and H_2O_2 in the Cell-ELISA. Rat-1 fibroblasts were grown in 96-well plates and treated with 50 µM H_2O_2 or 20 ng/ml PDGF for the indicated times. The phosphorylated forms of MAP kinase were determined with the polyclonal phospho-specific p44/p42^{MAPK} antibody. Relative increases in the phosphorylation of MAP kinase after treatment of cells were calculated as described in the above text and are depicted as arbitrary units. Results ± SEM of a representative experiment are shown (*n*=8).

Phosphorylation of MAP kinase induced by H_2O_2 in the Cell-ELISA is dose-dependent

Kinase activity assays showed that the activation of MAP kinase induced by H_2O_2 is dose-dependent.[6] To determine a dose-dependent phosphorylation of MAP kinase in the Cell-ELISA, Rat-1 fibroblasts were treated with increasing concentrations of H_2O_2 for 20 min. Total amounts of $p42^{MAPK}$ were used as an internal control for the amount of protein and were not affected by different treatments of the cells (data not shown). Relative increases in the phosphorylation of MAP kinase after treatment with H_2O_2 were calculated as described above. Figure 6 shows that the phosphorylation of MAP kinase induced by H_2O_2 significantly increased in a dose-dependent manner. Because this is in agreement with data obtained by kinase activity assays[6], this further supports the reliability of the Cell-ELISA as a method for screening the phosphorylation and thus activation of MAP kinase.

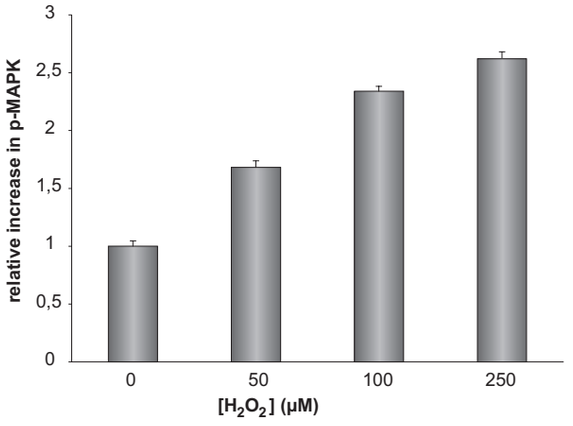

Fig. 6. Dose-dependent increase in the phosphorylation of MAP kinase in the Cell-ELISA. Rat-1 fibroblasts were cultured in 96-well plates and left untreated or treated with 50, 100 or 250 µM H_2O_2 for 20 min. The phosphorylated forms of MAP kinase were detected with the polyclonal phospho-specific $p44/p42^{MAPK}$ antibody. Increases in the phosphorylation of MAP kinase in H_2O_2-treated cells compared with untreated cells were calculated as described previously in the text and are depicted as arbitrary units. Results ± SEM of a representative experiment are shown (*n*=8).

Cell-ELISA for the phosphorylation of MAP kinase in myocardial cells

To show that the application of the Cell-ELISA to study the phosphorylation of MAP kinase is not restricted to fibroblast cells, the Cell-ELISA was performed in fully differentiated myocardial muscle cells as well. Therefore, cardiac myocytes were isolated from neonatal rat hearts[25] and seeded in 96-well plates as described in *Materials and Methods*. Treatment of these cells with 50 µM H_2O_2 for 20 min resulted in a significant increase in the phosphorylation of MAP kinase (Fig. 7), which is in agreement with previously published

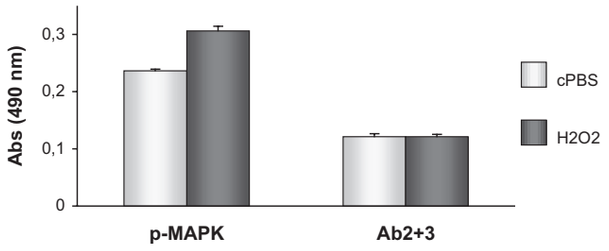

Fig. 7. Phosphorylation of MAP kinase induced by H_2O_2 in myocardial cells in the Cell ELISA. Myocardial cells were plated on 96-well plates and treated with 50 µM H_2O_2 for 20 min. After fixation and permeabilization of the cells, the phosphorylated forms of MAP kinase were detected with the polyclonal phospho-specific p44/p42^{MAPK} antibody. For background values, different wells were incubated with the biotinylated goat anti-rabbit antibody and with the streptavidin-PO (Ab2+3). Results ± SEM of a representative experiment are shown (*n*=8).

data.[29] Statistical analysis revealed that the total amount of p42^{MAPK}, which was used as a control for the amount of protein, remained unchanged after treatment of myocardial cells with H_2O_2 (data not shown). Figure 7 also shows that background values of the secondary biotinylated goat anti-rabbit antibody and the streptavidin-PO were not influenced by H_2O_2. After calculation of the amount of phosphorylated MAP kinase as described above, a significant increase in phosphorylation of ± 1.3-fold was found. It should be noticed that this increase is less pronounced as compared with the effects of H_2O_2 on the phosphorylation of MAP kinase in Rat-1 fibroblasts (Figs. 4-6). A reasonable explanation would be that myocardial cells have more efficient anti-oxidant defence mechanisms, compared with Rat-1 fibroblasts. This is supported by the fact that myocardial cells contain many peroxisomes and mitochondria and therefore need protection against damage to biological compounds by oxygen free radicals. Nevertheless, because Figure 7 shows a significant H_2O_2-induced increase in the phosphorylation of MAP kinase in myocardial cells, this indicates that the Cell-ELISA is applicable in various cell types.

Requirement of serum-starvation in the Cell-ELISA

Because certain compounds to be tested in the Cell-ELISA will be highly lipophilic, application of these compounds is only possible using a carrier such as serum components. In order to investigate whether an increase in the phosphorylation of MAP kinase can still be observed in cells that are serum-exposed, the Cell-ELISA was performed in Rat-1 cells that were grown in DMEM supplemented with serum. From these experiments it appeared that a significant increase in the phosphorylation of MAP kinase of approximately 2-fold could be obtained (data not shown). This shows that serum-starvation is not required for detection of

an increase in MAP kinase phosphorylation and that the Cell-ELISA is applicable for testing the effect of lipophilic compounds, using serum as a carrier.

Because the Cell-ELISA is applicable in both serum-exposed and serum-starved cells and in various cell types, it is concluded that the Cell-ELISA is a reliable method for detection of the phosphorylation of MAP kinase as a measure of its activity and can be used for screening the effectivity of chemical compounds or conditions that modulate cellular stress through activation of MAP kinase.

Concluding Remarks

In this study, we have developed a Cell-ELISA in 96-well plates to determine the phosphorylation of MAP kinase as a measure of its activity. For the development of this assay, the phospho-specific p44/p42^{MAPK} antibody was used, which specifically detects the phosphorylated forms of MAP kinase in a mixture of proteins. We showed that in this Cell-ELISA, the transient and dose-dependent phosphorylation of MAP kinase is similar to SDS-PAGE assays under proliferative or stress conditions.[6,7] Furthermore, we showed that the Cell-ELISA is applicable to different cell types and that serum starvation is not a requirement for detection of an increase in the phosphorylation of MAP kinase. From these experiments, we conclude that the Cell-ELISA is a reliable method for screening the phosphorylation, and thus the activation, of MAP kinase.

There are several advantages of this assay compared with other methods to determine the phosphorylation of MAP kinase:

1. The utilization of 96-well plates, which requires a relatively small number of cells and facilitates the screening of large number of compounds and measurements in replicate.
2. The utilization of relatively intact cells as the target of the assay instead of isolated proteins.
3. Time: in a relatively short time one can effectively conduct a large number of assays, using a microplate reader. Furthermore, most of the actions to be performed can be automated and therefore the Cell-ELISA could be modified for high throughput screening.
4. Simplicity: in contrast with electrophoretic mobility shift and SDS-PAGE, the Cell-ELISA does not require the separation of the phosphorylated and the non-phosphorylated forms of MAP kinase. Furthermore, the Cell-ELISA is a nonradioactive method to quantitatively determine the phosphorylation or activation of MAP kinase, which makes this assay preferable to kinase activity assays.

Because MAP kinase is phosphorylated and activated in response to cellular stress, the Cell-ELISA is applicable for screening the effectivity of chemicals that modulate different forms of stress. The ability of oxidants to induce the phosphorylation of MAP kinase as a

cellular response to oxidative stress can, for instance, be tested. On the other hand, it is possible to screen antioxidants in their efficiency to prevent or reduce this cellular response. It should be noticed that the application of our assay is not restricted to large-scale screening of compounds or conditions that modulate cellular stress, but can also be used for the screening of chemicals or compounds that induce cell proliferation or differentiation through the phosphorylation of MAP kinase.

Acknowledgements

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Application of the cellular phospho-MAPK assay

Adapted from: "Systemic mapping of reactive species-specific antioxidant efficacy and synergy in cultured Rat-1 fibroblasts"

Jan A. Post, Miriam Makkinje, Bart de Haan, Chris Th.W.M. Schneijdenberg, Frans van der Sman, Joanna Belkner, Greg P.C. Drummen, Renate de Wit, Eward H.W. Pap, C. Theo Verrips, Arie J. Verkleij and Philip J. Rijken (In prep.).

Introduction

Oxygen free radicals are produced during normal cell metabolism and can cause damage to DNA, lipids and proteins. Therefore, cells are equipped with various enzymes and compounds that function to protect the cell from oxidant damage. By scavenging of free radicals, both enzymatic and nonenzymatic antioxidants normally keep the cellular oxygen free radicals at low levels. The protecting role of small exogenous antioxidants, mostly derived from dietary fruits and vegetables, has gained wide scientific interest. Until now, elaborate systematic screenings on the functions and interactions of exogenous antioxidants have mostly been performed in cell-free systems. Research into dietary antioxidant functionality should, however, not only take into account the interactions of the exogenous antioxidants with each other. In addition, cellular uptake of dietary antioxidants and their interactions with endogenous antioxidant systems and cellular functioning should be included as well. Therefore, we have tested the functionality of various antioxidants in a cellular screening system. In this study, the phosphorylation of MAP kinase was used as a marker for oxidative stress and the efficacy of antioxidants in protection against H_2O_2-induced MAP kinase phosphorylation was studied using the Cell-ELISA as described in *chapter 2*.

Materials and Methods

Antioxidants used

The following antioxidants were used: α-tocopherol, γ-tocopherol, ascorbic acid (Merck, Darmstadt, Germany), lutein, lycopene, quercetin, chlorogenic acid (Sigma, St. Louis, MO), kaempferol (Fluka, Germany), tyrosol and hydroxytyrosol were synthesized as described (Baraldi et al., 1983). Palm oil carotenoids (a mixture of α- and β-carotene) was obtained from Biocon and both EGC and ECGC were obtained from Lipton (US). Hydrophilic antioxidants (ascorbic acid, tyrosol, hydroxytyrosol, EGC, ECGC, chlorogenic acid) were directly dissolved in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% fetal calf serum (FCS) (Gibco). Lipophilic antioxidants (kaempferol, quercetin, tocopherols) were dissolved in ethanol. Palm oil carotenoids, lutein and lycopene were dissolved in tetrahydrofurane. Lipophilic antioxidants were then added to 100% FCS (Gibco) and shaken under argon for 30 min at 37°C. Subsequently, 9 volumes of DMEM (Gibco) were added.

Cell-ELISA

Rat-1 fibroblasts were grown in 96-well plates (Nunc) in DMEM (Gibco) supplemented with 7.5% FCS (Gibco) in a 5% CO_2 humidified atmosphere at 37°C. Then, growth medium was removed and cells were incubated overnight with growth medium containing the antioxidants to be tested as described above. The next morning, cells were supplied with fresh antioxidants and incubated for another two hours. After washing with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.2) supplemented with 5 mM glucose

(PBS_{gluc}), cells were treated with H_2O_2 in PBS_{gluc} at 37°C for 10 min. Then, after fixation and permeabilization of the cells, the amount of phosphorylated p44/p42^{MAPK} was determined as described in *chapter 2*.

Results and discussion

First, dose response curves were obtained for all the antioxidants investigated in this study. Therefore, Rat-1 fibroblasts were grown in 96-well plates and overnight incubated with different antioxidants as described in *Materials and Methods*. The next morning, antioxidants were refreshed, followed by further incubation for 2 hr. Then, cells were washed and treated with 50 µM H_2O_2 for 10 min followed by detection of the amount of phosphorylated p44/p42^{MAPK}. Although exposure of cells to 50 µM H_2O_2 did not result in maximal phosphorylation of p44/p42^{MAPK} (Figure 6, chapter 2), sub-maximal responses were preferred so that potential positive and negative effects of antioxidants could be detected. Figure 1 shows, as an example, the dose response curves for α-tocopherol, γ-tocopherol and quercetin. Quercetin revealed to protect cells from H_2O_2-induced phosphorylation of p44/p42^{MAPK} in a relatively small concentration range. For the tocopherols, the protective range appeared broader, but less effective. Similar dose response curves were obtained with the other antioxidants and the effective antioxidant concentration varied between $5*10^{-8}$ – 10^{-5} M.

Next, the antioxidant concentration that gave a 50% inhibition of the H_2O_2-induced phosphorylation of p44/p42^{MAPK}, also referred to as IC_{50}, was determined. The results are

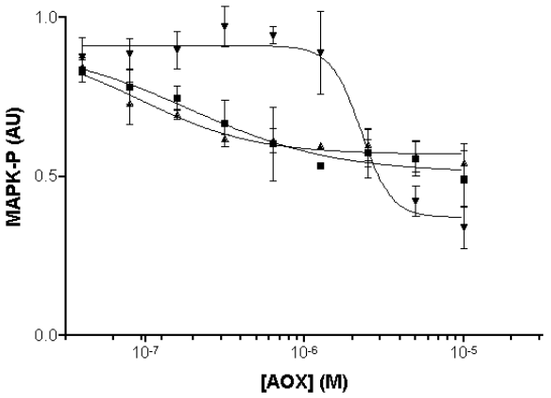

Fig.1 Dose response curves for the antioxidants (AOX) α-tocopherol (■), γ-tocopherol (▲) and quercetin (▼). Rat-1 fibroblasts were incubated overnight with increasing concentrations of AOX as described in *Materials and Methods*. The next morning, cells were supplied with fresh AOX and incubated for another two hr. Then, cells were washed with PBS_{gluc} and incubated with 50 µM H_2O_2 in PBS_{gluc} for 10 min. Thereafter, the amount of phosphorylated p44/p42^{MAPK} was determined as described in *Materials and Methods* of *Chapter 2*. Results ± SD of a representative experiment are shown ($2 \leq n \leq 3$).

listed in Table 1 and revealed that α-tocopherol, γ-tocopherol and quercetin were the most effective antioxidants, followed by kaempferol and hydroxytyrosol. Although the other antioxidants were taken up by the cells (data not shown), they did not show any protection as single compounds. When combinations of antioxidants were tested using the Cell-ELISA, additive effects of antioxidant capacity were observed. However, no synergistic antioxidant effects were detected and antioxidants that did not show any protection as single compounds also did not protect cells against H_2O_2-induced MAP kinase phosphorylation in combinations with other antioxidants.

Comparison of these results with the Trolox Equivalent Antioxidant Capacity (TEAC) values (Table 1) shows a different ranking of the antioxidant capacity. Based on the TEAC

Table 1. Measured EC_{50} and TEAC values for the antioxidants tested.

Antioxidant	*EC_{50} [µM]*	*TEAC [mM]*
α-tocopherol	0.13 ± 0.03	1.1 ± 0.01
γ-tocopherol	0.15 ± 0.03	1.1 ± 0.02
Quercetin	1.15 ± 0.23	4.7 ± 0.06
Kaempferol	2.75 ± 0.35	1.3 ± 0.08
Hydroxytyrosol	2.80 ± 0.39	0.73 ± 0.04
Palm oil carotenoids	No effect	3.4 ± 0.56
Lutein	No effect	1.5 ± 0.04
Lycopene	No effect	3.0 ± 0.1
Tyrosol	No effect	0.45 ± 0.02
EGCG	No effect	7.7 ± 1.11
ECG	No effect	8.3 ± 0.73
Chlorogenic acid	No effect	1.1 ± 0.08
Ascorbic acid	No effect	1.0 ± 0.02

TEAC values were adapted from reference 2

values, both quercetin and palm oil carotenoids were, for instance, expected to give a high cellular protection against H_2O_2-induced MAP kinase phosphorylation, whereas α-tocopherol and γ-tocopherol were expected to give a lower protection. However, testing those antioxidants in the Cell-ELISA revealed that α-tocopherol and γ-tocopherol antioxidant capacity was better than antioxidant capacity of quercetin, whereas the palm oil carotenoids gave no protection at all. These results indicate that there is no clear correlation between TEAC values and biological activity and therefore, it is important to test antioxidant capacity in various cellular screening assays and other biological systems.

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Chapter 3

Hydrogen peroxide inhibits Epidermal Growth Factor Receptor internalization in fibroblasts

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Free Radic. Biol. Med. **28**, 28-38 (2000)

Abstract

Several cellular signal transduction cascades are affected by oxidative stress. In this study, the effect of hydrogen peroxide (H_2O_2) on the endocytosis of the epidermal growth factor (EGF) receptor was investigated. Exposure of HER14 cells to H_2O_2 resulted in a concentration-dependent inhibition of EGF receptor internalization. Binding studies demonstrated that this H_2O_2-induced inhibition of internalization was not due to altered binding of EGF to its receptor. Addition of H_2O_2 at different time points during internalization showed that EGF receptor internalization was rapidly reduced, suggesting that one of the first steps in the internalization process is inhibited. In addition, H_2O_2 inhibited the internalization of a different receptor, the chicken hepatic lectin (CHL) receptor, in a concentration-dependent manner as well. Treatment of cells with another inducer of oxidative stress, cumene hydroperoxide, also resulted in a decreased internalization. Finally, we showed that H_2O_2 inhibited EGF-induced mono-ubiquitination of the EGF receptor pathway substrate clone 15, a process that normally occurs during EGF receptor endocytosis. These results clearly show that oxidative stress interferes with EGF signaling.

Introduction

Oxygen free radicals are generated under both normal and pathological circumstances and have been implicated in the pathogenesis of diseases such as atherosclerosis and cancer, as well as in aging and in some inflammatory disorders.[1-5] The involvement of free radicals in the carcinogenic process suggests that oxidative stress might have an effect on the intrinsic signal transduction cascades leading to cell division, by modification of signaling proteins. Indeed oxidative stress, induced by H_2O_2 or ultraviolet light, induces the phosphorylation and activation of several proteins that are involved in signal transduction. These include members of the mitogen-activated protein (MAP) kinase family,[6-9] the MAP kinase kinase (MEK1) [8], Raf-1 [8], Ras [10] and growth factor receptors, such as the platelet derived growth factor (PDGF) receptor [11] and the epidermal growth factor (EGF) receptor.[12-14] Although the mechanisms through which radicals accomplish these modifications of signal transduction proteins have not well been established, there are strong indications that increased phosphorylation of the EGF receptor by H_2O_2 is the result of an inactivation of tyrosine phosphatases.[13,15] This suggests that H_2O_2 has an inhibitory effect on cellular negative feedback mechanisms that attenuate growth factor-induced signal transduction.

Another mechanism to attenuate EGF-induced signaling is downregulation, which includes the internalization and subsequent degradation of activated EGF receptors. Upon stimulation of cells with EGF, the activated receptors are recruited towards specialized,

clathrin-coated regions in the plasma membrane, the coated pits.[16-18] After clustering of the receptors in these regions, the pits bud from the plasma membrane into the cytoplasm, thereby forming clathrin-coated vesicles. These vesicles are subsequently transported from the plasma membrane through the cytoplasm by microtubuli. After uncoating, the vesicles fuse with the early endosomes. Although many endocytosed integral membrane proteins recycle efficiently to the plasma membrane, the majority of EGF receptors are transported to the late endosomes and finally to the lysosomes, where degradation takes place.[19-21]

The above-described downregulation of activated receptors is important, because cellular transformation and tumor formation can ensue from the inability of cells to undergo ligand-induced endocytosis.[22,23] Here, we report the effect of oxidative stress on the internalization of the EGF receptor upon EGF stimulation. Oxidative stress, induced by H_2O_2, rapidly inhibits the internalization of the EGF receptor in HER14 fibroblasts in a concentration-dependent manner. In addition, we show that H_2O_2 also reduces endocytosis of the chicken hepatic lectin (CHL) receptor, a transmembrane receptor that mediates endocytosis of glycoproteins terminating with N-acetylglucosamine or other glucose-related structures.[24] Treatment of HER14 cells with another inducer of oxidative stress, cumene hydroperoxide, results in a decreased endocytosis of EGF as well, indicating that EGF receptor internalization might be inhibited under different oxidative stress conditions. Finally, we show that H_2O_2 inhibits the mono-ubiquitination of EGF receptor pathway substrate clone 15 (Eps15), a process normally occurring during endocytosis of the EGF receptor.

Materials and Methods

Materials

EGF receptor grade was obtained from Collaborative Research, Waltham, MA; ^{125}I was purchased from Amersham Pharmacia Biotech., Buckinghamshire, UK. Tetramethyl-rhodamine-conjugated EGF (EGF-Rhod) was obtained from Molecular Probes, Leiden, the Netherlands. Cy3 was a product of Amersham Life Science, Inc., Pittsburgh, USA and Sephadex G25 was purchased from Pharmacia. P-phenylene-diamine (PPD) was obtained from Sigma, St. Louis, MO. PVDF membrane was a product of Boehringer Mannheim, Germany and Protifar was obtained from Nutricia, Zoetermeer, the Netherlands. JAE14 cells (HER14 cells stably transfected with CHL receptor cDNA) and agialofetuin (AGF) were a kind gift of Dr. J.C. den Hartigh, Utrecht University, the Netherlands. The monoclonal antibodies against the EGF receptor and phosphotyrosine (PY20) were purchased from Transduction Laboratories, Lexington, KY, and rabbit polyclonal antibody against Eps15 (anti-Eps15RF99) was a kind gift of Dr. P.M.P. van Bergen en Henegouwen, Utrecht University, the Netherlands.

Cell culture

HER14 cells (NIH 3T3 cells stably transfected with human EGF receptor cDNA), and JAE14

cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% fetal calf serum (Gibco) in a 5 % CO_2 humidified atmosphere. Tissue culture flasks and dishes were from Nunc, Life Technologies, Breda, the Netherlands. All experiments were performed with a cell density of 40,000 cells/cm^2.

Confocal scanning laser microscopy

Agialofetuin (AGF) was conjugated to Cy3 (AGF-Cy3) as described by the manufacturer and separated from nonconjugated Cy3 using a sephadex G25 column. HER14 cells and JAE14 cells were cultured on glass coverslips. After washing with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.2) at 0°C, cells were incubated with 40 ng/ml EGF-Rhod or with 2 µg/ml AGF-Cy3 in PBS supplemented with 5 mM glucose (PBS_{gluc}) in the presence or absence of 1, 2 or 5 mM H_2O_2 for the indicated times at 37°C. Other cells remained at 0°C for 30 min to determine the staining pattern when internalization was prevented. Thereafter, cells were washed once with cold PBS, followed by fixation with 3.5% paraformaldehyde in PBS-0 (PBS without $CaCl_2$ and $MgCl_2$) for 45 min at room temperature. After washing with PBS-0, cells were embedded in 10% Mowiol 4-88, 25% glycerol, 100 mM Tris pH 8.5 containing 1 mg/ml PPD and analyzed by confocal scanning laser microscopy (CSLM) (Lasersharp mrc-500, Biorad, Hemel Hempstad, UK).

Lactate dehydrogenase release

The quantity of lactate dehydrogenase (LDH) released to cellular supernatants was measured by the decrease in absorbance at 340 nm, due to the LDH-dependent conversion of pyruvate and NADH to lactate and NAD^+, respectively.[25] The percentage LDH release was calculated from the units of LDH released into PBS_{gluc} from cells incubated with or without H_2O_2 divided by the total units of LDH that could be released from cells permeabilized with Triton X-100. Incubation of cells with or without H_2O_2 was followed by the addition of catalase (100 U/ml) to the PBS_{gluc} to remove the remaining H_2O_2 and to prevent its interference with pyruvate. Control experiments showed that catalase did not interfere with the measurement of LDH released.

^{125}I-EGF internalization assays

^{125}I-EGF was prepared by the chloramine-T method, specific activity varying between 250,000 and 600,000 cpm/ng as described previously.[26, 27] HER14 cells were cultured in 25 cm^2 tissue culture dishes. Cells were washed twice with ice-cold PBS at 0°C and exposed to ^{125}I-EGF (0.5; 2; 4 or 10 ng/ml) in PBS_{gluc} in the absence or presence of 1, 2, or 5 mM H_2O_2 for the indicated times at 37°C. To determine background values, other cells were treated equally at 0°C to prevent internalization for the longest incubation time. After washing twice with cold PBS, cells were treated with acid wash (125 mM NaCl, 25 mM HAc, pH 3.0) for 15 min at 0°C to remove surface membrane-bound ^{125}I-EGF. Subsequently, cells were washed with PBS at 0°C and dissolved in 1 M NaOH at 37°C. Radioactivity

was measured in a gamma-counter (Crystal 5412 Multi Detector Ria System, United Technologies, Packard, USA).

^{125}I-EGF binding assay

HER14 cells were washed twice with cold PBS at 0°C and incubated with ^{125}I-EGF (4 ng/ml) in PBS_{gluc} in the presence or absence of 1, 2 or 5 mM H_2O_2 at 0°C for the indicated times. After washing three times with cold PBS at 0°C, cells were dissolved in 1 M NaOH and radioactivity was determined by counting in a gamma-counter as described above.

Tyrosine phosphorylation and Western blotting

HER14 cells were grown on 10 cm^2 tissue culture dishes. Cells were washed once with PBS, followed by stimulation with EGF (4 ng/ml) in PBS_{gluc} in the presence or absence of 5 mM H_2O_2 for 30 min at 37°C, while control cells were incubated with PBS_{gluc} for 30 min at 37°C. Then, cells were washed once with ice-cold PBS and lysed in 40 µl Laemmli sample buffer. Proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membrane. Blots were blocked for 1 hr at room temperature in 5% bovine serum albumin (BSA) in TBST (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.05% [v/v] Tween-20) when PY20 antibody was used, and in 2% milk powder in PBST (PBS with 0.05% [v/v] Tween-20) when the anti-EGF receptor antibody was used. Blots were then incubated with primary antibody in 0.5% BSA in TBST for PY20 and in 0.5% milk powder in PBST for anti-Eps15 antibody for 1 hr at room temperature. After washing, blots were incubated for 1 hr at room temperature with secondary horseradish peroxidase-conjugated antibodies diluted in the same buffer as used for the primary antibodies. Proteins were detected using the chemiluminescence procedure (Renaissance, DuPont NEN, Boston, MA).

Immunoprecipitations

HER14 cells were grown in 75 cm^2 tissue culture dishes. Cells were washed once with PBS, followed by stimulation with EGF (40 ng/ml) in PBS_{gluc} in the presence or absence of 1 mM H_2O_2 for 10, 20 or 30 min at 37°C, while control cells were incubated with PBS_{gluc} for 30 min at 37°C. After washing twice with ice-cold PBS, cells were lysed in Radio-Immuno-Precipitation Assay (RIPA) buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton-X-100, 0.1% SDS, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM benzamidine, 10 mM NaF, 1 mM Na_3VO_4) for 10 min at 4°C. Supernatants were incubated with 25 µl of a 50% slurry of Protein A-sepharose in RIPA buffer for 1 hr at 4°C. Subsequently, 1 µg anti-Eps 15 antibody (rabbit polyclonal) was added to precleared cell lysates, followed by incubation for 2 hr at 4°C. Protein A-sepharose was added as described above and lysates were further incubated for 2 hr at 4°C. Immunoprecipitates were washed once with RIPA buffer, twice with high-salt buffer (20 mM Tris-HCL pH 7.4, 0.5 M NaCl, 1% Triton-X-100, 1 mM PMSF, 1 mM benzamidine, 1 mM Na_3VO_4) and finally once with low-salt buffer (20 mM Tris-HCL pH 7.4, 0.15 M NaCl, 1% Triton-X-100, 1 mM PMSF, 1 mM benzamidine, 1 mM Na_3VO_4) at 4°C.

Samples were boiled for 10 min in 40 µl Laemmli sample buffer and proteins were separated by 8% SDS-PAGE. Western blots were detected with rabbit anti-Eps15 antibody or with PY20 antibody as described above.

Results

Effect of H_2O_2 on the internalization of the EGF receptor in HER14 cells

In order to investigate the effect of oxidative stress on the internalization of the EGF receptor, HER14 cells were exposed to tetramethyl-rhodamine-conjugated EGF (EGF-Rhod) in the presence or absence of H_2O_2 for 20 and 30 min at 37°C. Then, cells were fixed as described in *Materials and Methods* and studied using confocal scanning laser microscopy (CSLM). Cells that remained on ice to prevent internalization only showed staining of the plasma membrane, both in the absence and in the presence of H_2O_2 (Fig. 1, upper panels). However, cells incubated with EGF-Rhod in the absence of H_2O_2 at 37°C, showed a punctate pattern throughout the cytoplasm after 20 min and a perinuclear staining after 30 min of incubation (Fig. 1, left panels). This suggests that under these conditions, EGF was internalized into the cells and transported from the plasma membrane toward the perinuclear region in vesicle-like structures. In the presence of 1 mM H_2O_2, EGF was internalized as well, because EGF-Rhod was observed inside the cells after 30 min of incubation. However, as compared to control cells, the internalized EGF in the presence of 1mM H_2O_2 appeared less abundant and plasma membrane labeling was more intense (Fig. 1, middle panels). In the presence of 2 mM H_2O_2, EGF-Rhod was rarely observed in punctate, vesicle-like structures and in the presence of 5 mM H_2O_2, no intracellular probe was observed (Fig. 1, middle and right panels). In the latter case, the staining pattern was comparable to the pattern obtained from cells that remained on ice, suggesting that the internalization was blocked at this concentration of H_2O_2. Therefore, these data suggest that H_2O_2 has an inhibitory effect on the internalization of the EGF receptor in HER14 cells in a concentration-dependent manner.

To ensure that experiments were performed using viable cells, the release of lactate dehydrogenase (LDH) during incubation with varying concentrations of H_2O_2 was determined as a measure of cell integrity. Treatment of HER14 fibroblasts for 60 min with concentrations of H_2O_2 up to 10 mM did not result in significant LDH release as compared with control cells (Fig. 2). When higher concentrations H_2O_2 were used, there was a concentration-dependent increase in LDH release. To ensure good cell viability, 5 mM H_2O_2 was the highest concentration that was used in the subsequent experiments.

Although the results obtained by CSLM suggest that H_2O_2 inhibits EGF receptor internalization, these results do not, however, elucidate whether the internalization or the intracellular transport of vesicles is inhibited by H_2O_2. Therefore, the effect of H_2O_2 on the internalization process of ^{125}I-EGF was determined. HER14 cells were incubated with

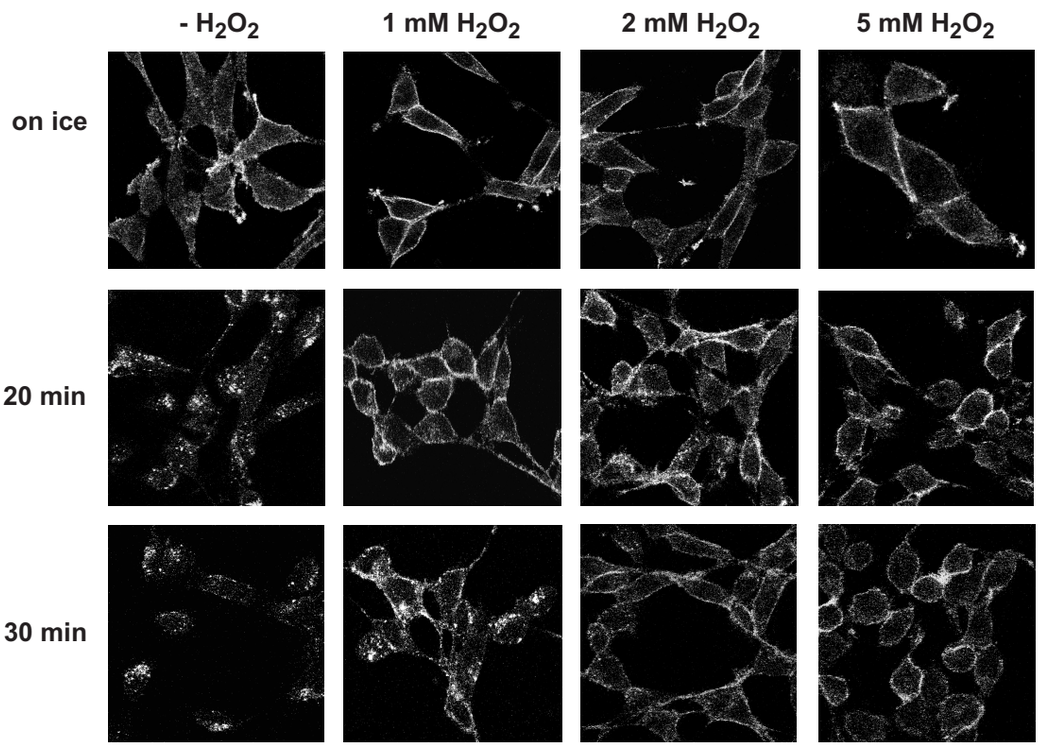


Fig. 1. H_2O_2-induced reduction of the internalization of EGF-Rhod. HER14 cells were washed with PBS and subsequently incubated with 40 ng/ml EGF-Rhod in the absence or presence of 1, 2 or 5 mM H_2O_2 for 20 and 30 min at 37°C or at 0°C (on ice) to prevent internalization. After fixation and embedding, cells were analyzed using CSLM and projections from the base to the top of the cells are shown.

different concentrations of ^{125}I-EGF in the presence or absence of H_2O_2 for 30 min at 37°C as described in *Materials and Methods*. Cells were then washed at low pH to remove EGF from non-internalized receptors and subsequently the internalized label was measured. Figure 3A shows that H_2O_2 reduced the internalization of EGF in a concentration-dependent manner. In the presence of 1 and 2 mM H_2O_2, some internalization still occurred. However, when cells were incubated with EGF in the presence of 5 mM H_2O_2, no significant increase in internalized label was observed, indicating that at this concentration of H_2O_2 the internalization of EGF was blocked. Figure 3A also shows that the decrease in internalization by H_2O_2 was independent of the concentration of EGF. In most cell types studied so far, the EGF receptor population consists of at least two classes. One class binds EGF with high affinity, whereas the other class binds EGF with low affinity.[28-30] The relative inhibition by

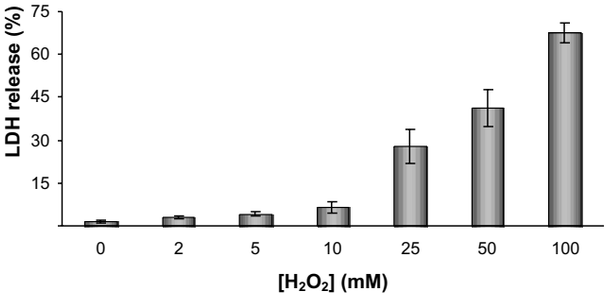


Fig. 2. Effect of increasing H_2O_2 concentrations on the release of LDH from HER14 cells. Cells were incubated with the indicated concentrations of H_2O_2 for 60 min at 37°C. Subsequently, LDH release was measured as described in *Materials and Methods*. Results +/- SEM are represented (*n* = 5).

different concentrations of H_2O_2 was almost identical at each concentration of EGF, suggesting that the inhibitory effect of H_2O_2 is not restricted to one class of EGF receptors. Therefore, the degree of inhibition of internalization is independent of the concentration of EGF, but dependent on the concentration of H_2O_2.

In order to determine whether H_2O_2 induces a block or a delay in the internalization of the EGF receptor, HER14 cells were incubated with 4 ng/ml ^{125}I-EGF at 37°C in the absence or presence of H_2O_2 for different time periods. The amount of internalized EGF was then determined as described in *Materials and Methods*. Figure 3B shows a rapid increase in the amount of internalized ^{125}I-EGF in the absence of H_2O_2. The amount of internalized label declined after 30 min of incubation, which is due to receptor downregulation and degradation of ^{125}I-EGF in the lysosomes, followed by the release of free ^{125}I.[31, 32] Indeed, ^{125}I, which could not be precipitated with trichloroacetic acid (TCA), appeared in the incubation medium after incubation of the cells for 30 and 60 min (not shown). In the presence of H_2O_2, the internalization of EGF was reduced in a concentration-dependent manner, which is comparable with the results shown in Figure 1. Furthermore, it is shown that the H_2O_2-induced decrease in the internalization was observed within 10 min of incubation and at every later time point (Fig. 3B). In the presence of 2 and 5 mM H_2O_2, there was no further increase in the amount of internalized EGF after incubations for longer than 30 min, suggesting that H_2O_2 does not induce a delay, but rather a concentration-dependent inhibition of EGF receptor internalization.

H_2O_2 rapidly inhibits EGF receptor internalization

To investigate whether H_2O_2 has an immediate effect on the internalization of EGF, time course experiments were performed in which H_2O_2 was added to cells after different periods of EGF incubation (Fig. 4). Thus, cells were first incubated with ^{125}I-EGF in the

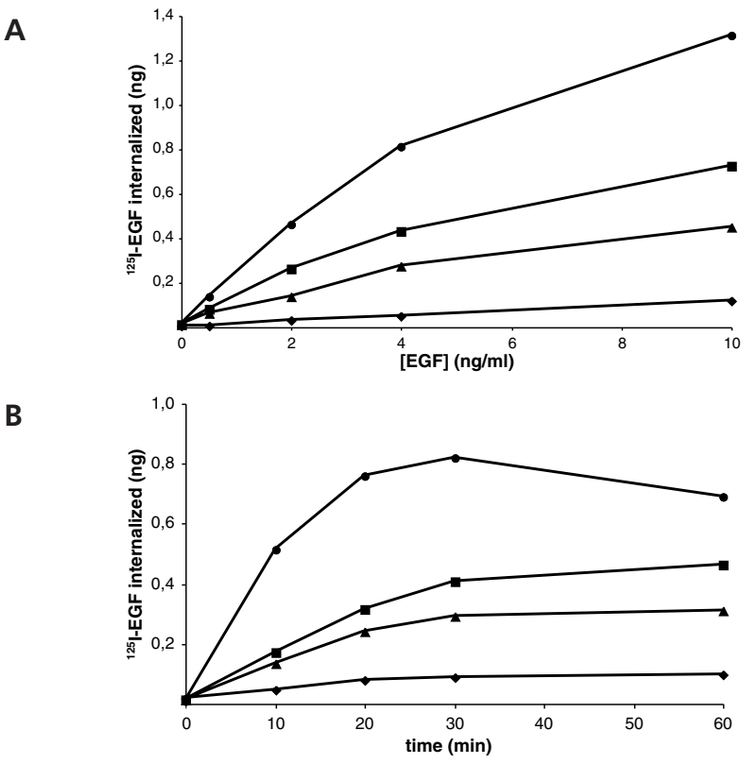


Fig. 3. Inhibitory effect of H_2O_2 on the internalization of ^{125}I-EGF. (A) HER14 cells were washed with ice-cold PBS and incubated with different concentrations of ^{125}I-EGF in the absence (●) or presence of 1 (■), 2 (▲), or 5 (◆) mM H_2O_2 for 30 min at 37°C. Then, cells were treated with acid wash for 15 min at 0°C to remove surface membrane-bound EGF and radioactivity was determined in a gamma-counter. Data of a representative experiment are shown. (B) HER14 cells were washed with ice-cold PBS and incubated with ^{125}I-EGF (4 ng/ml) in the absence (●) or presence of 1 (■), 2 (▲), or 5 (◆) mM H_2O_2 at 37°C as described in *Materials and Methods*. Subsequently, cells were treated with acid wash as described above, dissolved and radioactivity was determined by counting in a gamma-counter. Data of a representative experiment are shown.

absence of H_2O_2 for 10, 20 or 30 min, after which H_2O_2 (final concentration 5 mM) was added, followed by further incubation at 37°C. Thereafter, cells were washed at low pH to remove surface membrane-bound EGF and the internalized EGF was measured as described in *Materials and Methods*. Figure 4 shows that in the absence of H_2O_2 a rapid increase in the amount of internalized EGF was observed until 30 min, followed by a decline as described above. In the continuous presence of 5 mM H_2O_2, internalization of EGF was again blocked. Addition of H_2O_2 at 10 or 20 min caused a strong decrease in the amount of

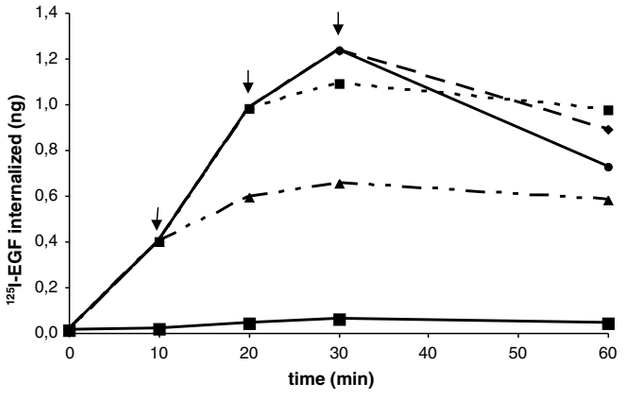


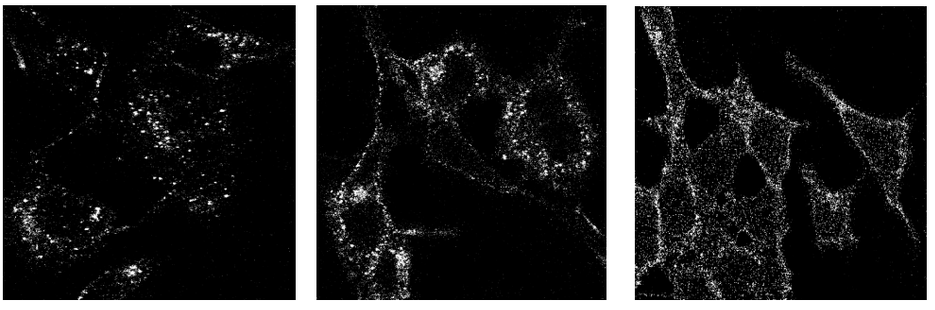
Fig. 4. Effect of H_2O_2 on the internalization of ^{125}I-EGF. HER14 cells were washed with ice-cold PBS and incubated with ^{125}I-EGF (4 ng/ml) in the absence of H_2O_2 (●), or in the continuous presence of 5 mM H_2O_2 (■) at 37°C. Other cells were first incubated in the absence of H_2O_2, followed by the addition of H_2O_2 (final concentration 5 mM) on t=10 (▲), t=20 (■) or t=30 (◆) respectively, after which cells were further incubated at 37°C. Then, cells were treated with acid wash for 15 min at 0°C and the internalized ^{125}I-EGF was measured by counting in a gamma-counter. Data of a representative experiment are shown.

internalized EGF during further incubation, as compared with control cells. This indicates that the internalization of the EGF receptor was rapidly inhibited after the addition of H_2O_2.

Another remarkable feature concerned the observation that, after addition of H_2O_2 at 10 or 20 min, almost no decrease was measured in the amount of internalized ^{125}I-EGF after incubations at 37°C for longer than 30 min, suggesting that H_2O_2 also has an inhibitory effect on the degradation of EGF. In conclusion, these experiments demonstrate that H_2O_2 inhibited the internalization of the EGF receptor rapidly after its addition to HER14 fibroblasts.

Inhibition of receptor-mediated endocytosis is not restricted to the EGF receptor or to H_2O_2

In order to investigate whether the inhibitory effect of H_2O_2 on endocytosis is specific to the EGF receptor, we determined the influence of H_2O_2 on the internalization of another transmembrane receptor, the CHL receptor. Therefore, the internalization of AGF-Cy3, a ligand of the CHL receptor, was studied by CSLM. Figure 5 demonstrates that after incubation for 30 min in the absence of H_2O_2, fluorescence-labeled AGF was observed inside the cells in punctate patterns, indicating that AGF was internalized. This internalization of AGF-Cy3 still occurred in the presence of 1 mM H_2O_2. However, in the presence of 5 mM H_2O_2, staining was observed primarily at the plasma membrane, again comparable to the staining pattern obtained from cells that remained on ice to prevent internalization (not



-H_2O_2 **1 mM H_2O_2** **5 mM H_2O_2**

Fig. 5. H_2O_2-induced inhibition of the internalization of AGF-Cy3. JAE14 cells were washed with ice-cold PBS and incubated with AGF-Cy3 (2 µg/ml) in the absence or presence of 1 or 5 mM H_2O_2 for 30 min at 37°C. Subsequently, cells were fixed as described in *Materials and Methods* and analyzed by CSLM. Optical sections through the center of the cells are shown.

shown). Therefore, it is concluded that H_2O_2 inhibits the endocytosis of the CHL receptor in a concentration-dependent manner as well.

To determine whether another inducer of oxidative stress also inhibits the internalization of the EGF receptor, HER14 cells were treated with EGF-Rhod in the presence or absence of cumene hydroperoxide for 30 min at 37°C. After fixation as described in *Materials and Methods*, cells were analyzed using CSLM. Incubation of cells with EGF-Rhod in the absence of cumene hydroperoxide resulted in a punctate, perinuclear staining (Fig. 6), indicating that EGF-Rhod was internalized and transported from the plasma membrane toward the perinuclear region in vesicle-like structures. However, cells incubated in the presence of cumene hydroperoxide showed primarily plasma membrane staining, comparable to the staining pattern obtained from cells that remained on ice to prevent internalization (Fig. 6). This shows that cumene hydroperoxide also has an inhibitory effect on the endocytosis of EGF-Rhod in HER14 cells.

Effect of H_2O_2 on the binding of EGF to its receptor

We have shown that the internalization of the EGF receptor is rapidly inhibited by H_2O_2 (Fig. 4). Therefore, it is likely that an early event of receptor-mediated endocytosis is affected. The inhibitory effect of H_2O_2 on internalization might be due to decreased binding of EGF to its receptor in the presence of H_2O_2, caused by a conformational change of either the EGF receptor or the EGF molecule itself. Therefore, the effect of H_2O_2 on EGF binding was determined. Cells were incubated with ^{125}I-EGF (4 ng/ml) in the presence or absence of H_2O_2 at 0°C to prevent internalization. After the cells were washed with ice-cold PBS, membrane-bound ^{125}I-EGF was measured as described in *Materials and Methods*. Figure 7

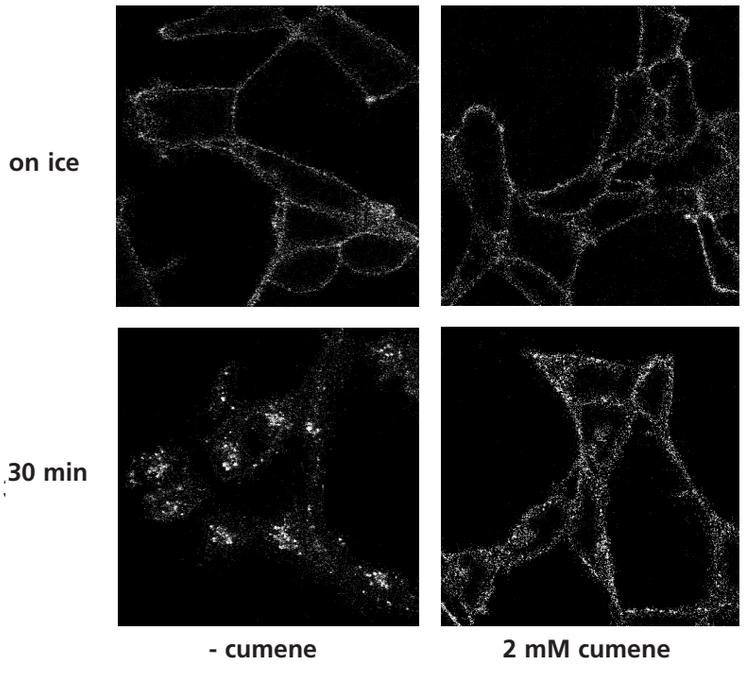


Fig. 6. Effect of cumene hydroperoxide on the internalization of EGF-Rhod. HER14 cells were washed with PBS and subsequently incubated with EGF-Rhod (40 ng/ml) in the absence or presence of 2 mM cumene hydroperoxide (cumene) for 30 min at 0°C (on ice) or for 30 min at 37°C. Then, cells were fixed and subsequently analyzed by CSLM. Optical sections through the center of the cells are shown.

demonstrates that H_2O_2 did not significantly influence the binding of EGF. Other experiments revealed that the binding of EGF at different concentrations (1 - 500 ng/ml) was not altered by H_2O_2 either (data not shown). Thus, the observed inhibition of EGF receptor internalization is not due to an effect of H_2O_2 on the binding of EGF to its receptor.

Effect of H_2O_2 on the EGF-induced tyrosine phosphorylation of the EGF receptor

To demonstrate that other early responses upon stimulation of cells with EGF are not inhibited, the influence of H_2O_2 on EGF-induced receptor phosphorylation was investigated. Therefore, HER14 fibroblasts were stimulated with either EGF or H_2O_2, or with the combination of H_2O_2 and EGF. Treatment of cells with EGF resulted in an increase in the tyrosine phosphorylation of the EGF receptor, whereas H_2O_2 induced a very small increase (Fig. 8). Clearly, the EGF-induced receptor phosphorylation was not inhibited in the presence of H_2O_2, and was even strongly enhanced. Densitometric analysis of the results of the blots

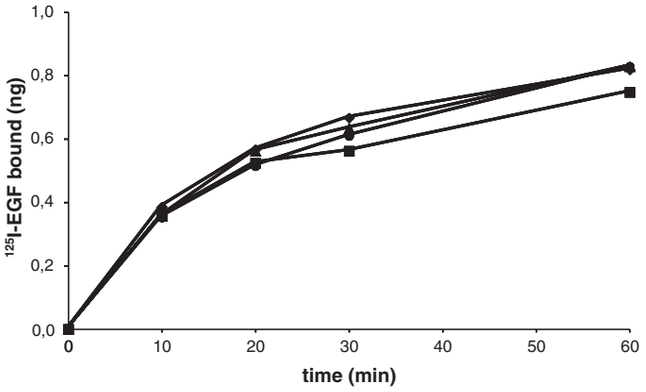


Fig. 7. Effect of H_2O_2 on the binding of EGF to its receptor. HER14 cells were washed with PBS at 0°C and subsequently incubated with ^{125}I-EGF (4 ng/ml) in the absence (●) or presence of 1 (■), 2 (▲), or 5 (◆) mM H_2O_2 at 0°C. After washing with ice-cold PBS, radioactivity was measured using a gamma-counter. Data of a representative experiment are shown.

of four independent experiments showed that the combination of EGF and H_2O_2 resulted in a 4.3-fold (± 0.9) higher phosphorylation of the EGF receptor, relative to the effect of EGF alone. Thus, although EGF receptor internalization is inhibited, H_2O_2 does not inhibit the tyrosine phosphorylation of the EGF receptor.

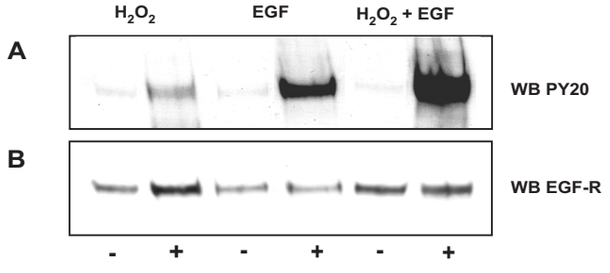


Fig. 8. Effect of H_2O_2 and EGF on the tyrosine phosphorylation of the EGF receptor. HER14 cells were washed with PBS and left untreated (-) or incubated with 5 mM H_2O_2 (+), 4 ng/ml EGF (+), or with 5 mM H_2O_2 and 4 ng/ml EGF (+) for 30 min at 37°C. Proteins from cell lysates were separated by 8% SDS-PAGE and Western blot was detected with PY20 antibody (A) or with anti EGF receptor antibody (B). A representative blot out of four independent experiments is shown.

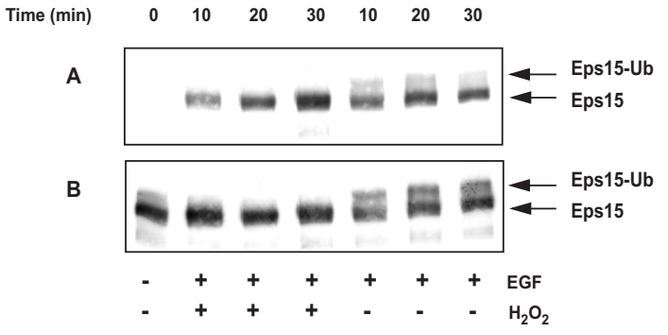


Fig. 9. Effect of H_2O_2 on the phosphorylation and mono-ubiquitination of Eps15. HER14 cells were washed with PBS and incubated with EGF (40 ng/ml) in the absence or presence of 1 mM H_2O_2 for the indicated times at 37°C. Subsequently, Eps15 was immunoprecipitated with polyclonal anti-Eps15 antibody as described in *Materials and Methods* and immunoprecipitates were separated by 8% SDS-PAGE. The phosphorylated forms of Eps15 were detected with monoclonal PY20 antibody (A) and Eps15 was detected with polyclonal anti-Eps15 antibody (B). The results of a representative experiment out of four independent experiments are shown.

H_2O_2 inhibits the mono-ubiquitination of Eps15 in HER14 cells

The experiments described above demonstrate that H_2O_2 rapidly inhibited the internalization of the EGF receptor and that this inhibition was not due to altered binding of EGF to its receptor in the presence of H_2O_2. One of the proteins that appears to be essential for EGF receptor endocytosis is the 142 kDa EGF receptor pathway substrate clone 15 (Eps15).[33] After stimulation of cells with EGF, Eps15 becomes transiently phosphorylated and mono-ubiquitinated.[34-36] It has been demonstrated that Eps15 ubiquitination, but not its phosphorylation, is inhibited under conditions that blocked internalization of the EGF receptor, such as low temperature, potassium depletion and hypertonic shock.[37] To establish whether H_2O_2 affects EGF-induced tyrosine phosphorylation or ubiquitination of Eps15, HER14 cells were stimulated with EGF in the presence or absence of H_2O_2, followed by immunoprecipitation of Eps15 as described in *Materials and Methods*. Figure 9A shows that the phosphorylation of Eps15 on tyrosine residues was induced by EGF, both in the presence and absence of H_2O_2. In the absence of H_2O_2, EGF induced a mobility shift of phosphorylated Eps15 within 10 min of incubation, as seen on Western blot by the appearance of a band of Eps15 of approximately 150 kDa. This was confirmed by detection of the same blot with anti-Eps15 antibody, which showed the presence of two bands of Eps15 after stimulation of cells with EGF for 10, 20 and 30 min (Fig. 9B). Previous studies have shown that this 8 kDa increase in molecular weight is the result of mono-ubiquitination of Eps15.[36] In contrast, after treatment of cells with EGF in the presence of 1 mM H_2O_2, the 150

kDa form of Eps15 could not be detected. This complete inhibition of EGF-induced ubiquitination of Eps15 by H_2O_2 was observed in four independent experiments. Therefore, although the phosphorylation on tyrosine residues remains unaffected, the mono-ubiquitination of Eps15 is inhibited by H_2O_2.

Discussion

In this study, the effect of H_2O_2 on the endocytosis of the EGF receptor was investigated through ligand-induced internalization studies. First, we examined the influence of H_2O_2 on the internalization of EGF-Rhod in HER14 cells. Data obtained by CSLM suggested that H_2O_2 reduced the internalization in a dose-dependent manner (Fig. 1). These findings were confirmed by ^{125}I-EGF-internalization studies, which revealed that in the presence of higher concentrations of H_2O_2, the internalization of EGF was abolished (Fig. 3). The inhibition of receptor-mediated endocytosis is not restricted to the EGF receptor and H_2O_2, because another inducer of oxidative stress, cumene hydroperoxide, also inhibits this process and furthermore, receptor-mediated endocytosis of the CHL receptor is inhibited by H_2O_2 as well (Figs. 5 and 6). Therefore, receptor-mediated endocytosis might be inhibited in general by oxygen free radicals, possibly by a general mechanism that is affected by oxidative stress.

This H_2O_2-induced inhibition of EGF receptor internalization is not due to cell death, because LDH release was negligible after treatment of HER14 cells for 60 min with concentrations of H_2O_2 up to 5 mM (Fig. 2). In addition, phosphorylation of the EGF receptor (Fig. 8) or, further downstream, of Eps15 (Fig. 9) and MAP kinase (not shown), were induced by EGF both in the absence and in the presence of H_2O_2. This demonstrates that, although EGF receptor internalization is inhibited, the cells are intact and early events in signal transduction are not blocked under the conditions used in the current study.

The concentrations H_2O_2 used to study the effect in HER14 cells are rather high. However, the HER14 cells are fairly resistant to oxidative stress and no loss of viability occurred during the experimental protocol, as discussed above. Cell number counts also showed that overnight recovery of the cells using the oxidative stress protocols of this study did not result in further cell death, except during recovery after 60 min of exposure to 5 or 10 mM H_2O_2 (results not shown). The effects of oxidative stress on receptor signaling as described in the present paper will most likely also occur at much lower concentrations of oxidative stress in cell types that show a higher sensitivity to oxidative stress.

We have shown that concurrent treatment of cells with EGF and H_2O_2 resulted in an enhanced tyrosine phosphorylation of the EGF receptor (Fig. 8). The oxidative stress-induced tyrosine phosphorylation of the EGF receptor has previously been suggested to be the result of an inactivation of tyrosine phosphatases, based on the assumption that the

receptor maintains spontaneous tyrosine kinase activity.[13] In addition, it has been shown that H_2O_2, produced in response to stimulation with EGF, reversibly inactivates protein-tyrosine phosphatase 1B in A431 cells.[15] Therefore, the enhanced phosphorylation observed in our experiments could be due to the inactivation of tyrosine phosphatases. Alternatively, inhibition of internalization, as found in the present study, might result in the inaccessibility of activated EGF receptors for cytosolic or endosome-associated tyrosine phosphatases.[38] This inaccessibility would also lead to enhanced receptor phosphorylation. In addition, an increase in the number of phosphorylated receptors might partly be due to H_2O_2-induced inhibition of internalization and, therefore, of degradation of phosphorylated (activated) receptors in the lysosomes.

As a result of an inhibition of the internalization by H_2O_2, the EGF receptors will not be degraded. Moreover, we have obtained strong evidence that the degradation of internalized EGF is inhibited by H_2O_2 as well (see Fig. 4; unpublished results). Thus, even when ^{125}I-EGF is internalized, its degradation is reduced in the presence of H_2O_2, because no free ^{125}I was detected in the incubation medium after incubation of cells for 30 or 60 min (data not shown). These results show that different stages of receptor-mediated endocytosis are inhibited by H_2O_2. However, future investigations may reveal whether the inhibition of receptor internalization and degradation of EGF by H_2O_2 is due to the same mechanism.

The inhibition of EGF receptor internalization by H_2O_2 occurs within minutes (Fig. 4), suggesting that internalization of the EGF receptor is inhibited at an early stage of the endocytotic process. Binding studies revealed that this inhibition is not due to altered binding of EGF to its receptor in the presence of H_2O_2 (Fig. 7). This is confirmed by the fact that pretreatment of either cells or EGF-Rhod with 5 mM H_2O_2 and subsequent removal of H_2O_2 by catalase did not inhibit internalization of EGF-Rhod (results not shown). This also indicates that, although H_2O_2 rapidly inhibits the internalization of the EGF receptor, pretreatment with H_2O_2 is not sufficient to accomplish this inhibition. Therefore, H_2O_2 might cause cellular changes that are rapidly reversible or H_2O_2 might influence a process involved in internalization that is induced specifically after activation of the receptor by its ligand.

EGF receptor pathway substrate Eps15 is involved in EGF receptor endocytosis and becomes phosphorylated and mono-ubiquitinated upon stimulation of cells with EGF.[33-36] We have shown that H_2O_2 inhibits the EGF-induced mono-ubiquitination of Eps15 (Fig. 9). Previous studies revealed that mono-ubiquitination of Eps15 was inhibited under different conditions that blocked receptor internalization.[37] Therefore, our results further support the observation that H_2O_2 indeed inhibits EGF receptor internalization.

A clear role of ubiquitination in receptor-mediated endocytosis has been recently shown.[39,40] Mutation of the ubiquitination sites on the α-factor receptor in yeast abolishes receptor endocytosis.[39] In mammalian cells, internalization of the growth hormone receptor is dependent on the ubiquitin conjugation system as well.[40,41] Furthermore, a rapid loss of

endogenous ubiquitin-protein conjugates and downregulation of ubiquitin-conjugating activity has been observed upon exposure of bovine retina cells or bovine lens epithelial cells to H_2O_2.[42,43] This suggests that the ubiquitination of proteins might be inhibited in general under the conditions used in our experiments. Therefore, we suggest that H_2O_2 might inhibit EGF receptor internalization by an inhibition of the ubiquitination of proteins involved in the internalization process. We are currently trying to determine the underlying mechanism involved in the inhibition of receptor internalization.

Acknowledgements

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Chapter 4

Large Scale Screening Assay to measure Epidermal Growth Factor Internalization

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Abstract

Recently, we showed that the internalization of the epidermal growth factor (EGF) receptor is inhibited by hydrogen peroxide (H_2O_2) in HER14 fibroblasts. In order to test the effect of various stress conditions on receptor internalization and to test a variety of antioxidants in their capacity to prevent or reduce the H_2O_2-induced inhibition of internalization, a screening assay was developed to measure the internalization in 96-well plates. In this assay, cells are exposed to biotin-conjugated EGF and the amount of internalized EGF is detected with horseradish peroxidase-conjugated streptavidin. We show that the results obtained by this new assay are comparable with those from internalization studies performed with radioactive labeled EGF. Therefore, the cellular internalization assay as presented here is a reliable method to measure EGF receptor internalization. Moreover, because elaborate processing of the cells is not required, the assay is a relatively fast and inexpensive method to study ligand-induced internalization in 96-well plates and thereby is suitable for large-scale screening of compounds or conditions interfering with this internalization.

Introduction

Oxygen free radicals have diverse effects on cell functioning, probably depending on both the dose and the duration of exposure. It has been well established that treatment of cells with hydrogen peroxide (H_2O_2) or ultraviolet light induces the phosphorylation and activation of several signaling proteins, including mitogen-activated protein (MAP) kinases,[1-3] $p21^{ras}$,[4] and the epidermal growth factor (EGF) receptor.[5-7] Phosphorylation of signaling proteins during oxidative stress would be accomplished by reversible inhibition of protein tyrosine phosphatases.[8,9]

Under normal conditions, ligand-induced activation and phosphorylation of the EGF receptor is followed by receptor downregulation, one of the mechanisms to attenuate EGF-induced signaling. After recruitment to clathrin-coated pits, the activated receptors are internalized and transported to the early endosomes. While the minor fraction may be recycled, the majority of the EGF receptors are transported to the late endosomes and to the lysosomes, where they becomes degraded.[10-15] The downregulation of activated receptors is apparently important, because the inability to undergo this ligand-induced endocytosis can lead to cellular transformation or tumor formation.[16,17]

Recently, ligand-induced internalization studies revealed that the internalization of the EGF receptor is inhibited in the presence of H_2O_2,[18] suggesting that the internalization might be inhibited by oxidative stress in general. In order to test the effect of a large variety of stress conditions on receptor internalization and to test various antioxidants in their

capacity to protect cells against the oxidative stress-induced inhibition of internalization, a screening assay was developed to quantitatively measure the internalization. Although internalization assays are available, they involve radioactive labeling of the ligand and/or extensive processing of the cellular material.[10,19] Therefore, we have developed a nonradioactive internalization assay in cells in 96-well plates that is partly based on a ligand binding assay as described by others[20] and partly on the cellular MAP kinase assay in 96-well plates as described previously.[3] In this assay, the internalized EGF is detected in cells without the requirement of multiple antibody incubations, and therefore the assay as presented in this study is a relatively rapid and inexpensive method to measure the internalization of the EGF receptor. Therefore, our internalization assay is preferable to other internalization assays and, because it can be easily adapted for automation, is suitable for large-scale screening.

Materials and Methods

Materials

Receptor-grade EGF was obtained from Collaborative Research, Waltham, MA; ^{125}I was purchased from Amersham Pharmacia Biotech., Buckinghamshire, UK. Biotin-conjugated EGF (EGF-biotin) was a product of Molecular Probes, Leiden, the Netherlands. Horseradish peroxidase-conjugated streptavidin (streptavidin-PO) was purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA. *O*-phenylene-diamine dihydrochloride (OPD) was obtained from ICN Biomedicals Inc., Aurora, OH. Bicinchoninic acid (BCA) reagent was purchased from Pierce, Rockford, IL.

Cell culture

HER14 cells (NIH 3T3 cells stably transfected with human EGF receptor cDNA) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% fetal calf serum (Gibco) in a 5 % CO_2 humidified atmosphere. Tissue culture flasks and dishes were from Nunc Life Technologies, Breda, the Netherlands.

^{125}I-EGF binding assay

^{125}I-EGF was prepared by the chloramine-T method, specific activity varying between 250,000 and 600,000 cpm/ng as described previously.[21,22] HER14 cells were cultured in 25 cm^2 tissue culture dishes to a cell density of 40,000 cells/cm^2. HER14 cells were washed twice with cold phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.2) at 0°C and incubated with ^{125}I-EGF (4 ng/ml) in PBS supplemented with 5 mM glucose (PBS_{gluc}) at 0°C for the indicated times. After washing 3 times with cold PBS at 0°C, cells were dissolved in 1 M NaOH during 1 hr at 37°C. Radioactivity was measured in a gamma-counter (Crystal^{TM} 5412 Multi Detector RIA System, United Technologies Packard, Meriden, CT).

^{125}I-EGF internalization assay

HER14 cells were cultured in 25 cm^2 tissue culture dishes to a cell density of 40,000 cells/cm^2. Cells were washed twice with ice-cold PBS at 0°C and exposed to ^{125}I-EGF (4 ng/ml) in PBS_{gluc} in the absence or presence of H_2O_2 for the indicated times at 37°C. To determine background values, other cells were treated identically at 0°C to prevent internalization for the longest incubation period. After washing twice with cold PBS, cells were treated with acid wash (125 mM NaCl, 25 mM HAc, pH 3.0) for 15 min at 0°C to remove surface membrane-bound ^{125}I-EGF. Subsequently, cells were washed with PBS at 0°C, then dissolved in 1 M NaOH during 1 hr at 37°C and radioactivity was determined by counting in a gamma-counter as described above.

EGF-biotin binding assay in 96-well plates

HER14 cells were cultured in 96-well plates to a cell density of approximately 80,000 cells/cm^2. Cells were washed twice with ice-cold PBS at 0°C and incubated with EGF-biotin (50 ng/ml) in PBS_{gluc} in the presence or absence of 100-fold molar excess of unlabeled EGF (5 µg/ml) for the indicated times at 0°C. After washing 3 times with cold PBS at 0°C, cells were fixed and permeabilized with 3.5% paraformaldehyde, 0.25% glutaraldehyde and 0.25% Triton X-100 in PBS without 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-0) for 30 min at 37°C. Next, cells were washed once with PBS-0, treated twice for 5 min with 50 mM glycin in PBS-0, and blocked with PBS-0 containing 2% gelatin and 0.05% (v/v) Tween-20 for 45 min at 37°C. After washing once with 0.2% gelatin in PBS-0, cells were exposed to streptavidin-PO diluted 1:15,000 in 0.2% gelatin in PBS-0 for 1 hr at 37°C. Extensive washing with 0.2% gelatin in PBS-0 for 30 min at 37 °C was followed by incubation with the horseradish peroxidase substrate OPD (3.7 mM) in 50 mM Na_2HPO_4 and 25 mM $C_6H_8O_7{\cdot}H_2O$ for 25 min in the dark at room temperature. The reaction was stopped by the addition of 50 vol% 1 M H_2SO_4 and spectrophotometric readings were performed at 490 nm using a Microplate Reader (Benchmark, Bio-Rad Laboratories, Inc., Hercules, CA). Using the Bio-rad software, data were exported into a spreadsheet for subsequent analysis.

EGF-biotin internalization assay in 96-well plates

HER14 cells were grown on 96-well plates (Nunc) to a cell density of approximately 80,000 cells/cm^2. After washing with PBS, cells were treated with EGF-biotin in the absence or presence of H_2O_2 in PBS_{gluc} at 0°C or at 37°C for the indicated times. Subsequently, cells were washed twice with ice-cold PBS at 0°C and treated with acid wash for 15 min at 0°C. After washing twice with cold PBS, total amounts of protein after different treatments were determined with protein assay reagent BCA as described by the manufacturer (Pierce, Rockford, IL), while cells in other wells were fixed and permeabilized with 3,5% paraformaldehyde, 0.25% glutaraldehyde, and 0.25% Triton X-100 in PBS-0 for 30 min at 37°C. Next, the amount of internalized EGF-biotin was detected as described for the EGF-biotin binding assay. To determine background absorbance values, cells were treated as described above, except for the incubations with EGF-biotin and streptavidin-PO.

Results and Discussion

Binding of EGF-biotin in 96-well plates

Because binding of EGF to its receptor is the first step in receptor-mediated endocytosis, we first tested the time-dependent binding of biotin-conjugated EGF (EGF-biotin) to the EGF receptor in 96-well plates and compared the results with those obtained by binding studies performed with ^{125}I-EGF. HER14 cells were incubated with ^{125}I-EGF or EGF-biotin for 10, 20, 30 or 60 min at 0°C to prevent internalization and to allow binding. Then, the amount of bound EGF was determined as described in *Materials and Methods*. Figure 1 shows a time-dependent increase in the binding of both ^{125}I-EGF (Fig. 1A) and EGF-biotin (Fig. 1B) to its receptor.

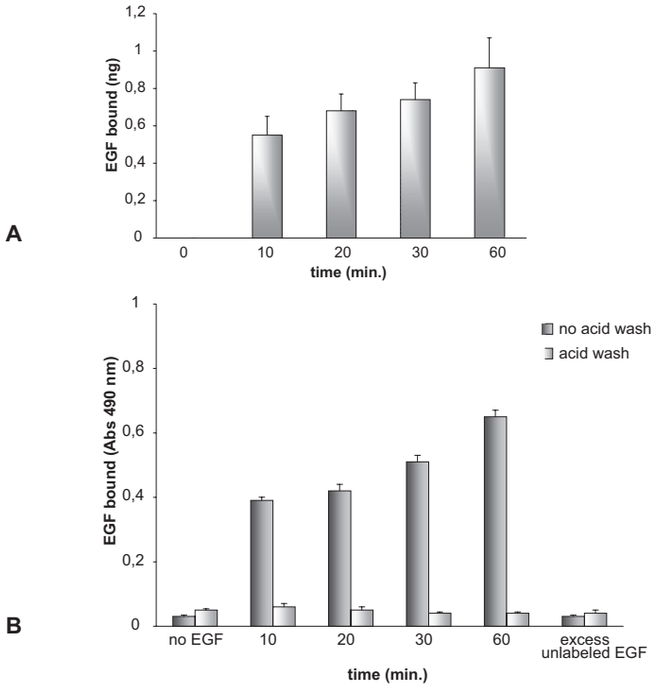

Fig. 1. Time-dependent binding of EGF. (A) HER14 cells were washed with ice-cold PBS and incubated with ^{125}I-EGF (4 ng/ml) for 10, 20, 30, or 60 min at 0°C. Then, cells were washed at 0°C and radioactivity was determined in a gamma-counter as described in *Materials and Methods.* Results +/- SEM of three independent experiments are shown. (B) HER14 cells were grown in 96-well plates and after washing with ice-cold PBS at 0°C, cells were left untreated (no EGF) or were incubated with EGF-biotin (50 ng/ml) for 10, 20, 30, or 60 min at 0°C, or for 60 min at 0°C with EGF-biotin (50 ng/ml) in the presence of 100-fold molar excess of unlabeled EGF (5 µg/ml) (excess unlabeled EGF). Subsequently, cell were left untreated (no acid wash), or were treated with acid wash (acid wash) for 15 min at 0°C and the amount of bound EGF-biotin was determined by measurement of the absorbance at 490 nm as described in *Materials and Methods.* Results +/- SEM of a representative experiment are shown (*n*=8).

To ensure that only EGF-biotin bound to non-internalized receptors was detected in the 96-well plate system, other wells were treated with acid wash after incubation of cells with EGF-biotin at 0°C for the indicated times. Subsequently, cells were fixed and the amount of bound EGF-biotin was detected by measuring the absorbance at 490 nm as described in *Materials and Methods*. As shown in Figure 1B, no increase in absorbance was observed after treatment of cells with acid wash as compared to control cells (no EGF), indicating that the internalization was completely inhibited at 0°C and showing that only EGF-biotin bound to non-internalized receptors was detected. Incubation of cells with EGF-biotin in the presence of an excess of unlabeled EGF resulted in an inhibition of binding of EGF-biotin, showing that EGF-biotin bound specifically to the EGF receptor. These results are comparable with the results obtained by others,[20] and therefore we considered this method reliable for the development of an internalization assay in 96-well plates.

Concentration-dependent internalization of the EGF receptor in 96-well plates

To measure the internalization of EGF-biotin, HER14 cells were cultured in 96-well plates and treated with different concentrations of EGF-biotin for 30 min at 37°C or 0°C. Thereafter, cells were treated with acid wash to remove surface membrane-bound EGF-biotin. Subsequent fixation and permeabilization of the cells was followed by incubation with streptavidin-PO, and the amount of internalized EGF-biotin was determined as described in *Materials and Methods*. Figure 2 shows that treatment of HER14 cells with increasing concentrations of EGF-biotin for 30 min at 37°C resulted in increased amounts of internalized EGF-biotin, which is in agreement with previously published data.[18] Background values were

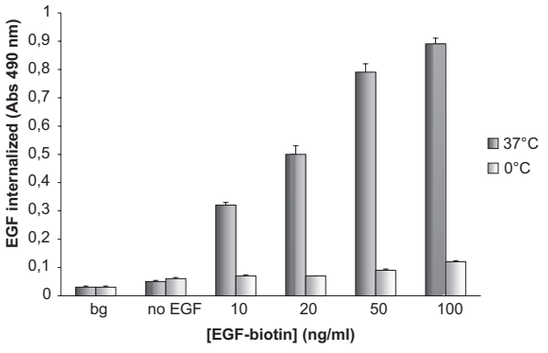

Fig. 2. Dose-dependent increase in the amount of internalized EGF-biotin. HER14 cells were grown in 96-well plates and after washing with PBS, cells were left untreated (no EGF) or were treated with increasing concentrations of EGF-biotin for 20 min at 37°C or 0°C. Subsequently, cells were treated with acid wash for 15 min at 0°C and the amount of internalized EGF-biotin was detected as described in *Materials and Methods*. To determine background values, both incubations of cells with EGF-biotin and streptavidin-PO were omitted (bg). Results +/- SEM of a representative experiment are shown (*n*=8).

obtained from cells treated in an identical manner, except for the incubations with EGF-biotin and streptavidin-PO (Fig. 2, bg). As shown in Figure 2, absorbance values of non-stimulated cells (no EGF) were not significantly increased as compared with background absorbance values (bg), indicating that there was hardly any nonspecific binding of streptavidin-PO.

To confirm that only internalized EGF-biotin was measured, HER14 cells in other wells were incubated with increasing concentrations of EGF-biotin for 60 min at 0°C to allow binding and to prevent internalization. Subsequent detection of the amount of internalized EGF-biotin after treatment of these cells with acid wash revealed that hardly any increase in the amount of internalized EGF-biotin was detected as compared to control cells (no EGF). This shows that surface membrane-bound EGF-biotin was indeed effectively removed by treatment with acid wash and that only internalized EGF-biotin was detected. Therefore, these results show that a concentration-dependent increase in EGF receptor internalization after incubation with EGF-biotin at 37°C can be detected using the presently described assay.

Time-dependent internalization of the EGF receptor in 96-well plates

To further validate the newly developed assay for determination of EGF receptor internalization, we compared time-dependent internalization of EGF-biotin in 96-well plates with results obtained by internalization studies performed with ^{125}I-EGF. HER14 cells were treated with ^{125}I-EGF or with EGF-biotin for 10, 20, 30 or 60 min at 37°C as described in *Materials and Methods*. As shown in Figure 3A, treatment of cells with ^{125}I-EGF induced an increase in the amount of internalized EGF during the first 30 min of incubation. This was followed by a decrease, as a result of degradation of ^{125}I-EGF in the lysosomes.[10] Measuring the internalization of EGF-biotin in 96-well plates also resulted in a time-dependent increase in internalization, as shown by an increased absorbance at 490 nm (Fig. 3B). Compared to the results obtained with ^{125}I-EGF, the decrease in internalized EGF-biotin after 30 min was less abundant. This was probably due to a difference in degradation kinetics of EGF-biotin as compared to ^{125}I-EGF, or to a difference in retention of the label. Despite this difference, both assays showed a time-dependent internalization pattern with an optimum at 30 min. Treatment of nonstimulated cells with streptavidin-PO did not influence background values (not shown), which is comparable with results shown in Figure 2. Furthermore, treatment of cells with acid wash after incubation with EGF-biotin on ice again revealed that surface membrane-bound EGF was removed (not shown), and therefore only internalized EGF-biotin was detected.

To exclude that EGF-biotin was nonspecifically internalized, cells were treated with EGF-biotin in the absence or presence of 100-fold molar excess of unlabeled EGF for 30 min at 37°C. This was again followed by treatment of the cells with acid wash and detection of the amount of internalized EGF-biotin by measuring the absorbance at 490 nm. Treatment of

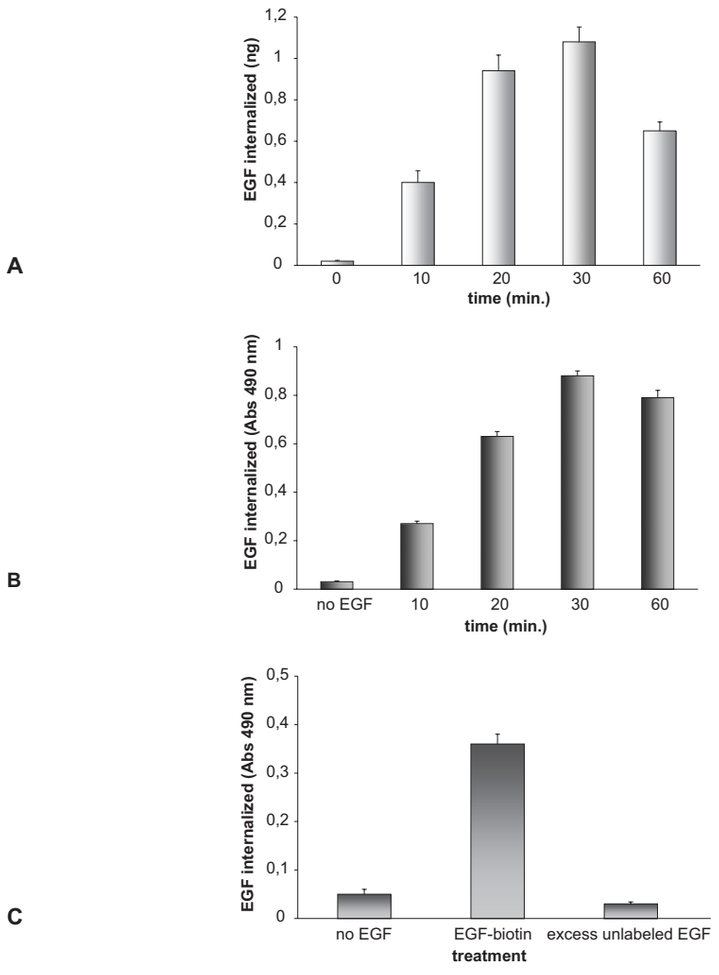

Fig. 3. Time-dependent increase in the amount of internalized EGF. (A) HER14 cells were washed with ice-cold PBS and incubated with ^{125}I-EGF (4 ng/ml) for 10, 20, 30, or 60 min at 37°C. Then, cells were treated with acid wash for 15 min at 0°C and radioactivity was determined in a gamma-counter as described in *Materials and Methods*. Results +/- SEM of seven independent experiments are shown. (B) HER14 cells were grown in 96-well plates and after washing with PBS, cells were left untreated (no EGF) or were incubated with EGF-biotin (50 ng/ml) for 10, 20, 30, or 60 min at 37°C. Subsequently, cells were treated with acid wash for 15 min at 0°C. After fixation and permeabilization of the cells, the amount of internalized EGF-biotin was determined as described in *Materials and Methods*. Results +/- SEM of a representative experiment are shown (n=8). (C) HER14 cells were grown in 96-well plates and after washing with PBS, cells were left untreated (no EGF) or were incubated with EGF-biotin (50 ng/ml) in the absence (EGF-biotin) or presence of excess unlabeled EGF (5 µg/ml) (excess unlabeled EGF) for 30 min at 37°C. After fixation and permeabilization, the amount of internalized EGF-biotin was determined as described in *Materials and Methods*. Results +/- SEM of a representative experiment are shown (*n*=8).

cells with EGF-biotin in the presence of excess unlabeled EGF showed no internalization of EGF-biotin (Fig. 3C), indicating that EGF-biotin was specifically internalized upon interaction with the EGF receptor. All these data indicate that the internalization assay in 96-well plates is a reliable method to determine the amount of internalized EGF.

Hydrogen peroxide inhibits EGF receptor internalization in HER14 cells in 96-well plates

Previously performed studies revealed that H_2O_2 inhibits the internalization of the EGF receptor in HER14 cells in a concentration-dependent manner.[18] To further support the reliability of the internalization assay in 96-well plates, the effect of H_2O_2 on the internalization in this assay was determined and results were again compared with results

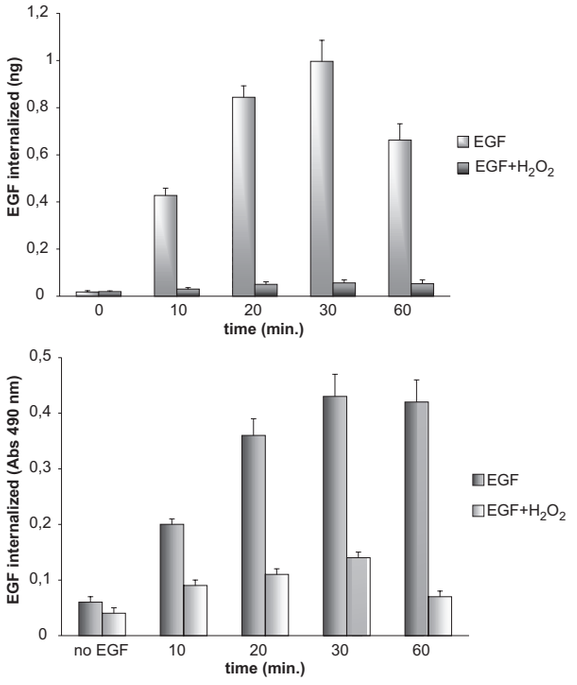

Fig. 4. H_2O_2 inhibits the internalization of ^{125}I-EGF and EGF-biotin in 96-well plates. (A) HER14 cells were washed with ice-cold PBS and incubated with ^{125}I-EGF (4 ng/ml) in the absence or presence of 5 mM H_2O_2 for 10, 20, 30, or 60 min at 37°C. After treatment of cells with acid wash for 15 min at 0°C, radioactivity was determined in a gamma-counter. Results +/- SEM of four independent experiments are shown. (B) HER14 cells were grown in 96-well plates, washed with PBS, and left untreated (no EGF) or incubated with EGF-biotin (50 ng/ml) in the absence or presence of 5 mM H_2O_2 for 10, 20, 30, or 60 min at 37°C, followed by treatment with acid wash as described above. After fixation and permeabilization of the cells, the amount of internalized EGF-biotin was determined as described in *Materials and Methods.* Results +/- SEM of a representative experiment are shown (*n*=8).

obtained from internalization studies performed with ^{125}I-EGF. Both assays showed a time-dependent increase in EGF receptor internalization in the absence of H_2O_2 and again, a difference in degradation kinetics or retention of both probes was observed (Fig. 4). However, in the presence of 5 mM H_2O_2, the internalization of both ^{125}I-EGF and EGF-biotin was inhibited, which is in agreement with previously described studies.[18] H_2O_2 did not influence background values (not shown) or nonspecific binding of streptavidin-PO (no EGF), as shown in Figure 4B. The incomplete inhibition of internalization of EGF-biotin in the presence of 5 mM H_2O_2 might be due to the relatively high cell density as compared to cell densities used in internalization assays performed with ^{125}I-EGF. Other studies indeed revealed that the sensitivity of cells to oxidative stress decreased with increasing cell density (unpublished results).

Comparison of the EGF-biotin internalization in Figures 3B and 4B shows a difference in absolute absorbance values. This interexperiment variability is most likely caused by variation in the cell number as observed by protein analysis (not shown). However, the data shown in these figures are qualitative comparable.

To demonstrate that the inhibition of EGF-biotin internalization in the presence of H_2O_2 as shown in Figure 4B was not due to differences in total amounts of proteins after treatment of cells, other wells were incubated with EGF-biotin in the absence or presence of H_2O_2 for 30 min at 37°C as well, followed by treatment with acid wash and determination of total amounts of proteins per well as described in *Materials and Methods*. In the presence of H_2O_2, internalization of EGF-biotin was again inhibited as compared with cells treated with EGF-biotin in the absence of H_2O_2 (Table 1). Determination of the total amounts of proteins revealed that treatment of cells with H_2O_2 did not significantly influence total amounts of proteins (Table 1), indicating that inhibition of internalization by H_2O_2 was not due to loss of cellular proteins by degradation or cell lysis.

To test whether H_2O_2 inhibited the internalization of EGF-biotin in a concentration-dependent manner, HER14 cells were grown in 96-well plates and stimulated with EGF-biotin

Table 1. Determination of the amount of internalized EGF-biotin and the total amount of proteins after treatment with EGF-biotin (50 ng/ml) in the absence or presence of 5 mM H_2O_2 for 30 min at 37˚C.

	Average ± SD	
Type of treatment	*Internalized EGF (Abs 490 nm)*	*Total amount of protein (µg)*
No EGF-biotin	0.08 ± 0.03	13.01 ± 1.89
EGF-biotin	0.52 ± 0.11	12.24 ± 1.46
EGF-biotin + H_2O_2	0.17 ± 0.02	12.89 ± 0.68

The values represent the mean ± SD with n=8.

in the absence or presence of 1, 2, 5 and 10 mM H_2O_2 for 30 min at 37°C. Determination of the amount of internalized EGF-biotin showed that H_2O_2 indeed inhibited the internalization in a concentration-dependent manner (Fig. 5), which is in agreement with previously performed studies.[18] Besides showing the mean +/- SEM, we also included the individual

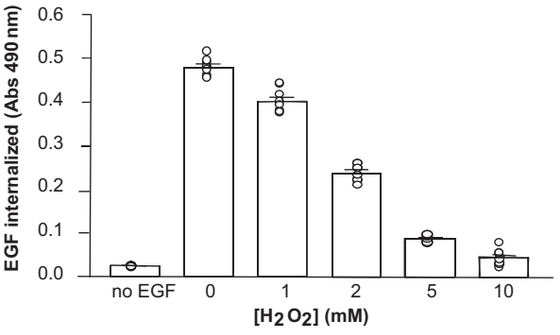

Fig. 5. Dose-dependent inhibition of the internalization of EGF-biotin by H_2O_2 in 96-well plates. HER14 cells were grown in 96-well plates and after washing with PBS, cells were left untreated (no EGF) or were treated with EGF-biotin (50 ng/ml) in the absence or presence of 1, 2, 5, or 10 mM H_2O_2 for 30 min at 37°C, which was followed by treatment of cells with acid wash for 15 min at 0°C. The amount of internalized EGF-biotin was then determined by measuring the absorbance at 490 nm as described in *Materials and Methods*. Individual data points are shown as circles.Results +/- SEM of a representative experiment are shown (*n*=8).

data points in Figure 5. This shows the robustness of the assay and clearly shows that there is no need for eight replicates in future screenings. Control experiments again revealed that only internalized EGF-biotin was detected and that treatment of cells with different concentrations of H_2O_2 did not influence total amount of proteins (not shown). Therefore, these results show that the internalization assay in 96-well plates is a reliable and sensitive method to measure gradual changes in the internalization of the EGF receptor.

Concluding Remarks

In this study, we have developed a reliable nonradioactive assay for measuring the internalization of the EGF receptor induced by EGF-biotin in 96-well plates. Time-dependent and concentration-dependent internalization of EGF-biotin was detected using this assay, and we showed that the results were comparable with results obtained with previously performed methods.[10,18] Furthermore, we showed that the internalization of EGF-biotin was inhibited by H_2O_2 and that this inhibition was not due to loss of total amounts of proteins. Using this internalization assay, concentration-dependent inhibition of internalization by H_2O_2

was detected, which was in agreement with previously published data[18] and shows that the assay is sensitive enough to measure gradual changes in internalization.

There are several advantages of this assay as compared with other methods to determine EGF receptor internalization. First of all, detection of internalization in 96-well plates requires only a relatively small number of cells and EGF-biotin, and facilitates the screening of large numbers of compounds that might interfere with receptor internalization. Furthermore, utilization of 96-well plates facilitates measurements in replicate and, because most of the actions to be performed can be automated, the assay could be easily modified for large-scale screening. Another advantage of the internalization assay is that intact cells are used as the targets, which implies that there is no necessity for further elaborate processing of the cells. Furthermore, because the internalized EGF-biotin is detected in one step, utilization of antibodies is not required. Therefore, the assay as presented here is a relatively fast and inexpensive method to measure EGF receptor internalization and this makes the assay preferable to other related nonradioactive, ELISA-based methods.

The newly developed internalization assay allows a rapid, nonradioactive and qualitative assessment of EGF internalization to screen the effect of drugs or experimental conditions on this internalization. Although ^{125}I-EGF is preferred for determination of the absolute amount of internalized EGF, the internalization assay performed with EGF-biotin allows quantification of the degree of inhibition. Moreover, because of the absence of radioactivity, the internalization assay performed with EGF-biotin can be performed in any lab equipped with a multi-well reader, without special precaution for radioactive work.

We showed that the internalization of the EGF receptor was inhibited in the presence of H_2O_2. Using the internalization assay as described in this study, we are able to determine the effects of many other stress conditions that might influence the internalization of the EGF receptor. Furthermore, various antioxidants can be tested for their ability to protect cells against the H_2O_2-induced inhibition of receptor internalization.

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Chapter 5

Hydrogen peroxide reversibly inhibits Epidermal Growth Factor (EGF) receptor internalization and coincident ubiquitination of the EGF receptor and Eps15

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Abstract

Recently, we demonstrated that hydrogen peroxide (H_2O_2) inhibits the internalization of the epidermal growth factor (EGF) receptor and the EGF-induced mono-ubiquitination of EGF receptor pathway substrate clone 15 (Eps15) in fibroblasts. In addition, it was suggested that EGF receptor internalization might be inhibited by H_2O_2 by inhibition of ubiquitination of proteins involved in endocytosis. Here, we show that H_2O_2 also inhibits the poly-ubiquitination of the EGF receptor in fibroblasts. Furthermore, recovery of the cells resulted in re-establishment of ubiquitination of both the EGF receptor and Eps15 and coincided with restoration of internalization of those receptors that had bound EGF in the presence of H_2O_2. In addition, EGF receptor internalization was inhibited by the sulphydryl reagent N-ethylmaleimide (NEM), indicating that intact SH groups might be required for receptor-mediated endocytosis. Furthermore, H_2O_2 rapidly induced an increase in the cellular ratio of GSSG:GSH and removal of H_2O_2 resulted in a fast restoration of the ratio of GSSG:GSH. Therefore, these results suggest a relation between the inhibition of internalization, ubiquitination and an increase in GSSG:GSH ratio and strengthen the hypothesis that H_2O_2 inhibits EGF receptor internalization by an inhibition of ubiquitination of proteins involved in EGF receptor-mediated endocytosis.

Introduction

Oxygen free radicals are generated under both normal and pathological circumstances and have been implicated in the pathogenesis of diseases such as atherosclerosis and cancer, as well as in aging and in some inflammatory disorders.[1-5] In the last few years, the involvement of oxygen free radicals in intrinsic signal transduction pathways leading to cell division has been studied in great detail. It has been demonstrated that extracellular addition of inducers of oxidative stress, such as hydrogen peroxide (H_2O_2), can induce the phosphorylation and activation of several proteins that are involved in these signaling pathways.[6-13] Moreover, recent studies revealed that H_2O_2 is produced upon stimulation of cells with Epidermal Growth Factor (EGF)[14] or Platelet-derived Growth Factor (PDGF).[15,16] It has been suggested that oxygen free radicals act as second messengers in signal transduction upon stimulation of cells with growth factors and are involved in increased receptor phosphorylation.[14,16,17] This increased phosphorylation of signaling proteins by H_2O_2 is probably accomplished by a reversible inactivation of tyrosine phosphatases, via oxidation of essential sulphydryl groups within their active site cysteines.[17,18]

Recently, we have found that H_2O_2 inhibits the internalization of the EGF receptor in fibroblasts in a concentration-dependent manner.[19,20] The internalization and subsequent degradation of activated EGF receptors is one of the negative feedback mechanisms to

attenuate EGF-induced signaling. This downregulation is important, since the inability of cells to undergo ligand-induced receptor-mediated endocytosis might lead to cellular transformation or tumor formation.[21,22]

Although EGF receptor internalization was inhibited by H_2O_2, the binding of EGF to its receptor was not affected.[19] In addition, it was demonstrated that EGF-induced tyrosine phosphorylation of the EGF receptor was not inhibited in the presence of H_2O_2, indicating that the EGF-induced early events that occur prior to internalization were not inhibited.[19] Interestingly, we found that although the EGF-induced tyrosine phosphorylation of EGF receptor pathway substrate clone 15 (Eps15) was not affected, its mono-ubiquitination was inhibited in the presence of H_2O_2.[19] Thus far, a role of ubiquitination in EGF receptor-mediated endocytosis has not been established. However, recent studies revealed that the ubiquitination of both Eps15 and the EGF receptor occur at the plasma membrane in cells that are blocked in EGF receptor-mediated endocytosis by overexpressing mutated dynamin.[23] Moreover, a role of ubiquitination in the endocytosis of other receptors has been described.[24-26] Therefore, we have proposed that H_2O_2 might inhibit EGF receptor internalization by an inhibition of ubiquitination of proteins involved in EGF receptor-mediated endocytosis.[19]

A rapid and dose-dependent loss of endogenous ubiquitin-protein conjugates has been observed upon exposure of bovine retina cells or bovine lens epithelial cells to H_2O_2.[27,28] Furthermore, ubiquitination enzyme activities were shown to be reversibly inhibited by H_2O_2 and were proposed to be regulated by the cellular ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH).[28,29]

Here, we report the effect of H_2O_2 and recovery upon H_2O_2 removal on EGF receptor internalization, on the poly-ubiquitination of the EGF receptor and the mono-ubiquitination of Eps15. In addition, the cellular ratio of GSSG:GSH was determined upon treatment of fibroblasts with H_2O_2 and the effect of stress removal and subsequent recovery was studied as well. We found that re-establishment of ubiquitination and of increased GSSG:GSH levels coincided with re-establishment of EGF receptor internalization. Therefore, the data shown in this study further support the hypothesis that H_2O_2 inhibits EGF receptor internalization by an inhibition of ubiquitination of proteins involved in the internalization process.

Materials and Methods

Materials

EGF receptor grade was obtained from Collaborative Research, Waltham, MA; Biotin-conjugated EGF (EGF-biotin) was a product of Molecular Probes, Leiden, the Netherlands. Horseradish peroxidase-conjugated streptavidin (streptavidin-PO) was purchased from Jackson ImmunoResearch

Laboratories Inc., West Grove, PA. *O*-phenylene-diamine dihydrochloride (OPD) was obtained from ICN Biomedicals Inc., Aurora, OH. Bicinchoninic acid (BCA) reagent was purchased from Pierce, Rockford, IL. Protifar was obtained from Nutricia, Zoetermeer, the Netherlands. PVDF membrane and NADPH were products of Boehringer Mannheim GmbH, Germany; triethanolamine (TEA), 5,5-dithiobis-[2-nitrobenzoic acid] (DTNB), N-ethylmaleimide (NEM) and GSSG-reductase were obtained from Sigma, St. Louis, MO; metaphosphoric acid (HPO_3) was a product from Fluka Chemika, Neu-Ulm, Germany, and 2-vinylpyridine was obtained from Merck, Hohenbrunn, Germany. The monoclonal antibodies against the EGF receptor and phosphotyrosine (PY20) were purchased from Transduction Laboratories, Lexington, KY. The monoclonal anti-EGF receptor clone 528 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. The rat polyclonal antibody against HA was obtained from Boehringer Mannheim GmbH, Germany and horse radish peroxidase-conjugated secondary rabbit anti-mouse (RAM-PO), goat anti-rabbit (GAR-PO) and donkey anti-rat (DARa-PO) antibodies were from Jackson ImmunoResearch Laboratories Inc., West Grove, PA. The rabbit polyclonal antibody against Eps15 (anti-Eps15RF99) and HUB1 cells (HER14 cells stably transfected with cDNA encoding HA-tagged ubiquitin) were a kind gift of Dr. P.M.P. van Bergen en Henegouwen, Utrecht University, the Netherlands.

Cell culture

HER14 cells (NIH 3T3 cells stably transfected with human EGF receptor cDNA), and HUB1 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 7.5% fetal calf serum (Gibco) in a 5 % CO_2 humidified atmosphere. Tissue culture flasks and dishes were from Nunc, Life Technologies, Breda, the Netherlands.

Immunoprecipitations and Western blotting

HUB1 cells were grown in 75 cm^2 tissue culture dishes to a cell density of approximately 60,000 cells/cm^2. Cells were washed twice with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.2) supplemented with 5 mM glucose (PBS_{gluc}), followed by stimulation with EGF (50 ng/ml) in PBS_{gluc} in the absence or presence of 5 mM H_2O_2 for the indicated times at 37°C, while control cells were incubated with PBS_{gluc} for the longest incubation time at 37°C. For recovery, cells were washed twice with ice-cold PBS at 0°C after pre-incubation with EGF in the presence of 5 mM H_2O_2, followed by further incubation with PBS_{gluc} at 37°C for the indicated times. After washing twice with ice-cold PBS, Eps15 and EGF receptor were immunoprecipitated with 1 µg anti-Eps15 antibody (rabbit polyclonal) or with 1 µg anti-EGF receptor clone 528 antibody (monoclonal) respectively as described previously.[19,30] Immunoprecipitates were boiled for 10 min in 40 µl Laemmli sample buffer and proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membrane. Blots were blocked for 1 hr at room temperature in 2% milk powder in PBS without 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-0) containing 0.05% (v/v) Tween-20 (PBST). Then, blots were incubated with primary antibody (rat anti-HA antibody diluted 1:2000, rabbit anti-Eps15 antibody diluted 1:4000, or

mouse anti-EGF receptor antibody diluted 1:2000) in 0.5% milk powder in PBST for 1 hr at room temperature. After washing, blots were incubated for 1 hr at room temperature with secondary horseradish peroxidase-conjugated antibodies diluted in the same buffer as used for the primary antibodies. Proteins were detected using the chemiluminescence procedure (Renaissance, DuPont NEN, Boston, MA).

EGF-biotin internalization assay in 96-well plates

HER14 cells were cultured in 96-well plates to a cell density of approximately 80,000 cells/cm^2. After washing with PBS_{gluc}, cells were treated with EGF-biotin (50 ng/ml) in the absence or presence of the indicated concentrations of H_2O_2, NEM or DTNB in PBS_{gluc} at 37°C for the indicated times. For recovery, cells were washed twice with ice-cold PBS at 0°C after pre-incubation with EGF in the absence or presence of 2 or 5 mM H_2O_2, followed by further incubation with PBS_{gluc} for 10, 20 or 30 min at 37°C. Subsequently, the quantity of lactate dehydrogenase (LDH) released to cellular supernatants was determined as described previously.[19] Cells were washed twice with ice-cold PBS at 0°C and treated with acid wash (125 mM NaCl, 25 mM HAc, pH 3.0) for 15 min at 0°C. After washing twice with cold PBS, total amounts of protein after different treatments were determined with protein assay reagent BCA as described by the manufacturer (Pierce, Rockford, IL), while the amount of internalized EGF-biotin in cells in other wells was determined as described previously.[20]

^{125}I-EGF internalization assays

^{125}I-EGF was prepared by the chloramine-T method, specific activity varying between 250,000 and 600,000 cpm/ng as described previously.[31,32] HER14 cells were cultured in 25 cm^2 tissue culture dishes to a cell density of approximately 60,000 cells/cm^2. Cells were washed twice with ice-cold PBS at 0°C and exposed to ^{125}I-EGF (4 ng/ml) in PBS_{gluc} in the absence or presence of 1, 2, or 5 mM H_2O_2 for 10 min at 37°C. After washing twice with cold PBS, cells were treated with acid wash for 15 min at 0°C. Subsequently, cells were washed with PBS at 0°C and dissolved in 1 M NaOH at 37°C. Radioactivity was measured in a gamma-counter (Crystal^{TM} 5412 Multi Detector Ria System, United Technologies Packard, Meriden, CT).

Determination of reduced and oxidized glutathione

HER14 cells were cultured in 25 cm^2 tissue culture dishes to a cell density of approximately 60,000 cells/cm^2. Cells were washed twice with PBS_{gluc}, followed by treatment with 1, 2 or 5 mM H_2O_2 in PBS_{gluc} for the indicated times at 37°C, while control cells were incubated with PBS_{gluc} for the longest incubation time at 37°C. For recovery, cells were washed twice with ice-cold PBS after preincubation with 5 mM H_2O_2, followed by further incubation with PBS_{gluc} at 37°C for the indicated times. Then, a modified Tietze-recycling assay was used.[33-35] Briefly, cells were extracted with 3.33% metaphosphoric acid (HPO_3), containing 2.7 mM ethylene diaminetetraacetic acid (EDTA) and after centrifugation for 5 min at 8500 rpm at 4°C, 200 µl of the supernatant was mixed with 24 µl of 4 M triethanolamine (TEA). Then,

one part of the sample was used for determination of the total amount of glutathione [(GSH) and glutathione disulfide (GSSG)]. For determination of the amount of GSSG, another part of the supernatant containing 0.4 M TEA was incubated with 0.02 M 2-vinylpyridine for 1 hr at room temperature. Subsequently, 100 µl of a 0.1 M sodiumphosphate-buffer pH 7.5 containing 300 µM NADPH, 1.5 U/ml GSSG reductase and 225 µM DTNB was added to 50 µl of the samples and spectrophotometric readings were performed at 415 nm using a Microplate Reader (Benchmark, Bio-Rad Laboratories, Inc., Hercules, CA). Using the Bio-rad software, data were exported into a spreadsheet for subsequent analysis.

Results

H_2O_2 inhibits the poly-ubiquitination of the EGF receptor

H_2O_2 has been demonstrated to inhibit EGF receptor internalization in fibroblasts in a concentration-dependent manner with a complete inhibition in the presence of 5 mM H_2O_2.[19] Although this is a relatively high concentration of H_2O_2, HER14 cells are fairly resistant to oxidative stress and no loss of cell viability occurred during the experimental protocol, as discussed previously.[19] In addition, it was demonstrated that EGF-induced mono-ubiquitination of Eps15, a protein that plays an essential role in EGF receptor-mediated endocytosis,[36] was inhibited in the presence of H_2O_2.[19]

Stimulation of cells with EGF induces the poly-ubiquitination of the EGF receptor as well.[37] Because this ubiquitination most likely occurs at the plasma membrane and thus prior to internalization,[23] it was interesting to study the effect of H_2O_2 on this poly-ubiquitination of the EGF receptor. Therefore, HUB1 cells - HER14 cells expressing HA-ubiquitin - were

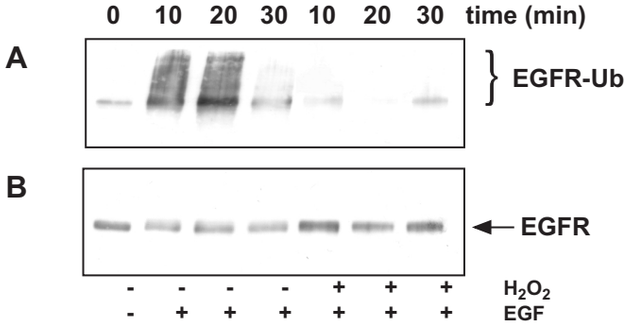


Fig. 1. Effect of H_2O_2 on the poly-ubiquitination of the EGF receptor. HUB1 cells were washed with PBS and incubated with EGF (50 ng/ml) in the absence or presence of 5 mM H_2O_2 for 10, 20 or 30 min at 37°C. Subsequently, EGF receptor was immunoprecipitated with monoclonal anti-EGF receptor antibody as described in *Materials and Methods* and immunoprecipitates were separated by 8% SDS-PAGE. The ubiquitinated EGF receptors were detected with polyclonal anti-HA antibody (A) and EGF receptor was detected on the same blot with monoclonal anti-EGF receptor antibody (B). The results of a representative experiment out of four independent experiments are shown.

treated with EGF in the absence or presence of 5 mM H_2O_2 for 10, 20 or 30 min, followed by immunoprecipitation of the EGF receptor. Detection on Western blot using anti-HA antibody (Fig. 1A) demonstrated the appearance of several bands with a molecular weight ≥ 170 kDa after stimulation of cells with EGF in the absence of H_2O_2 for 10 and 20 min, which disappeared after 30 min of incubation. This indicates that EGF induced a transient poly-ubiquitination of the EGF receptor, which is in agreement with previously performed studies.[23] Detection with monoclonal anti-EGF receptor antibody, directed against amino acids 996-1022 of the intracellular domain, showed only one band (Fig. 1B). Apparently, this antibody did not recognize the ubiquitinated form of the EGF receptor. Figure 1A also shows that the poly-ubiquitination of the EGF receptor was completely inhibited after stimulation of cells for 10, 20 and 30 min in the presence of 5 mM H_2O_2. Detection of the same blot with anti-EGF receptor antibody (Fig. 1B) revealed that differences in the amount of ubiquitinated EGF receptors were not due to differences in the amount of precipitated EGF receptors. Therefore, H_2O_2 inhibits EGF-induced poly-ubiquitination of the EGF receptor under the same conditions that blocked EGF receptor internalization.[19]

H_2O_2 reversibly inhibits the internalization of the EGF receptor

To investigate whether EGF receptor-mediated endocytosis recovered upon removal of H_2O_2, we used the non-radioactive screening assay to measure EGF receptor internalization as described previously.[20] Therefore, HER14 cells were cultured in 96-well

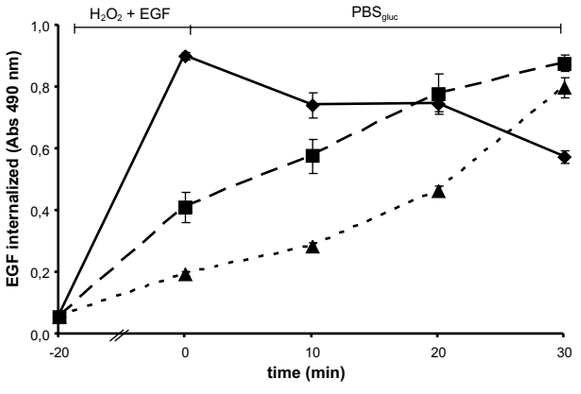


Fig. 2. Effect of H_2O_2 removal on EGF receptor internalization. HER14 cells were grown in 96-well plates and after washing with PBS, cells were incubated with EGF-biotin (50 ng/ml) in the absence (◆) or presence of 2 (■) or 5 (▲) mM H_2O_2 in PBS_{gluc} for 20 min at 37°C. Then, cells were washed twice with PBS at 0°C, followed by recovery in PBS_{gluc} for 10, 20 or 30 min at 37°C. This was followed by treatment with acid wash and after fixation and permeabilization of the cells, the amount of internalized EGF-biotin was determined as described in *Materials and Methods*. Results +/- SEM of a representative experiment are shown (*n*=8).

plates and treated with EGF-biotin in the absence or presence of 2 or 5 mM H_2O_2 for 20 min at 37°C. Subsequently, EGF-biotin and H_2O_2 were removed and cells were further incubated with PBS_{gluc} for 10, 20 or 30 min, followed by washing the cells at low pH to remove EGF-biotin from non-internalized receptors. After fixation and permeabilization, cells were incubated with streptavidin-PO and the amount of internalized EGF-biotin was determined. Figure 2 shows that treatment of cells with H_2O_2 for 20 min without recovery resulted in a concentration-dependent inhibition of the amount of internalized EGF. This was in agreement with previously performed data, which revealed that the internalization was inhibited by H_2O_2 at least until 60 min of incubation.[19,20] Further incubation with PBS_{gluc} of cells that were pre-incubated with EGF-biotin in the absence of H_2O_2 resulted in a time-dependent decline in the amount of internalized EGF-biotin, probably due to degradation of EGF-biotin in the lysosomes.[38,39] However, recovery of the cells that were pre-incubated in the presence of H_2O_2 resulted in a time-dependent re-establishment of EGF receptor internalization (Fig. 2). This indicated first of all that H_2O_2 did not irreversibly inhibit EGF receptor-mediated endocytosis and secondly that those receptors that had bound EGF-biotin during the pre-incubation in the presence of H_2O_2 became internalized upon removal of the stress. Furthermore, although recovery started within 10 min upon H_2O_2 removal, the time of complete restoration was dependent on the concentration of H_2O_2 that was used before removal of the stress. Recovery after treatment of cells with 2 mM of H_2O_2 resulted in an almost complete re-establishment of EGF receptor internalization within 20 min, whereas recovery after treatment with 5 mM H_2O_2 reached control levels (no H_2O_2) approximately after 30 min of recovery.

Ubiquitination of the EGF receptor and Eps15 are reversibly inhibited by H_2O_2

In a previous study, we proposed that the inhibition of EGF receptor internalization by H_2O_2 was due to an inhibition of ubiquitination. Recovery of EGF receptor internalization upon H_2O_2 removal should therefore be consistent with re-establishment of ubiquitination. To determine the reversibility of the inhibitory effect of H_2O_2 on the ubiquitination of the EGF receptor, HUB1 cells were treated with EGF in the absence or presence of 5 mM H_2O_2 for 20 min. This was followed by removal of the stress and recovery in PBS_{gluc} for 10, 20 or 30 min. Immunoprecipitation of the EGF receptor and subsequent detection of the Western blot with anti-HA antibody revealed that EGF-induced EGF receptor ubiquitination was again inhibited in the presence of H_2O_2 (Fig. 3A), whereas no difference in total amount of EGF receptors was observed (Fig. 3B). However, upon removal of H_2O_2 and further incubation in PBS_{gluc}, recovery of the poly-ubiquitination of the EGF receptor started within 10 min and was comparable with control levels (EGF) approximately after 20 min of recovery (Fig. 3A).

Previous studies revealed that EGF-induced mono-ubiquitination of Eps15 was inhibited by H_2O_2 after stimulation of cells for 10, 20 and 30 min.[19] In order to establish the

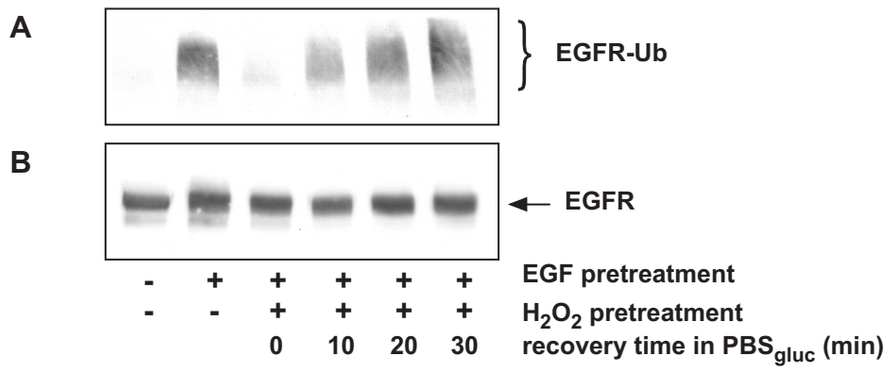


Fig. 3. Effect of H_2O_2 removal on the poly-ubiquitination of the EGF receptor. HUB1 cells were washed with PBS and incubated with EGF (50 ng/ml) in the absence or presence of 5 mM H_2O_2 in PBS_{gluc} for 20 min at 37°C. Thereafter, cells were washed twice with PBS at 0°C, followed by further incubation in PBS_{gluc} for 10, 20 or 30 min at 37°C. Subsequently, EGF receptor was immunoprecipitated with monoclonal anti-EGF receptor antibody as described in *Materials and Methods*. Immunoprecipitates were separated by 8% SDS-PAGE and ubiquitinated EGF receptors were detected with polyclonal anti-HA antibody (A) whereas EGF receptor was detected on the same blot with monoclonal anti-EGF receptor antibody (B). The results of a representative experiment out of three independent experiments are shown.

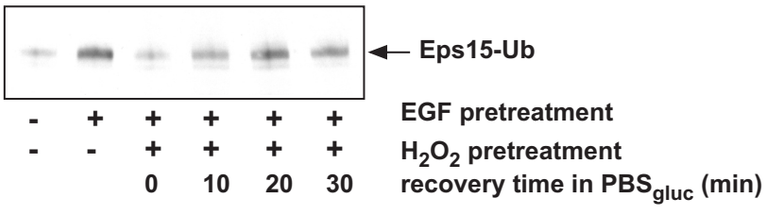


Fig. 4. Effect of H_2O_2 removal on the mono-ubiquitination of Eps15. HUB1 cells were washed with PBS and subsequently incubated with EGF (50 ng/ml) in the absence or presence of 5 mM H_2O_2 in PBS_{gluc} for 20 min at 37°C. Then, cells were washed twice with PBS at 0°C, followed by recovery in PBS_{gluc} for 10, 20 or 30 min at 37°C. Thereafter, Eps15 was immunoprecipitated with polyclonal anti-Eps15 antibody and immunoprecipitates were separated by 8% SDS-PAGE. The ubiquitinated form of Eps15 was detected with polyclonal anti-HA antibody. The results of a representative experiment out of three independent experiments are shown.

reversibility of the inhibition of ubiquitination of Eps15, HUB1 cells were incubated with EGF in the absence or presence of 5 mM H_2O_2 for 20 min, again followed by recovery in PBS_{gluc} for 10, 20 or 30 min. Subsequently, Eps15 was immunoprecipitated and, although there was no difference in the total amount of Eps15 (not shown), detection of the Western blot with anti-HA antibody revealed that H_2O_2 inhibited EGF-induced mono-ubiquitination of Eps15 (Fig. 4). Upon H_2O_2 removal, the inhibition of ubiquitination of Eps15 recovered and

quantification and correction for the total amount of Eps15 on the same blot revealed that recovery for 10, 20 or 30 min resulted in a re-establishment of the ubiquitination of Eps15 to 30%, 90% and 60% of control EGF (Fig. 4, lane 2) respectively. This indicates that control levels were almost reached upon 20 min after removal of the stress and, since Eps15 becomes transiently ubiquitinated upon EGF stimulation, this suggests that a 100% recovery is achieved around 20 min upon H_2O_2 removal. In conclusion, these data demonstrate that H_2O_2 reversibly inhibited the ubiquitination of both the EGF receptor and Eps15 and showed that re-establishment of the ubiquitination of these proteins started within 10 min and reached control levels approximately upon 20 min of recovery.

Effect of sulphydryl reagents on EGF receptor internalization

Because oxidative stress alters the cellular redox (and SH) status, the effect of the membrane-permeable thiol blocking agent N-ethylmaleimide (NEM), that irreversibly replaces the hydrogen atom in SH groups, on the internalization of the EGF receptor was investigated. In addition, to discriminate between the role of intracellular and extracellular SH groups in NEM action, the effect of the membrane-impermeant SH reagent 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB)[40] on EGF receptor internalization was determined as well. Therefore, HER14 cells were treated with EGF-biotin in the absence or presence of increasing concentrations of NEM or DTNB for 20 min at 37°C, followed by determination of the amount of EGF internalized as described previously.[20] Whereas treatment with DTNB did not have an effect on the internalization, NEM inhibited the amount of internalized EGF in a concentration-dependent manner (Fig. 5). This suggests that EGF receptor internalization is dependent on intracellular SH groups, whereas extracellular SH groups are not involved.

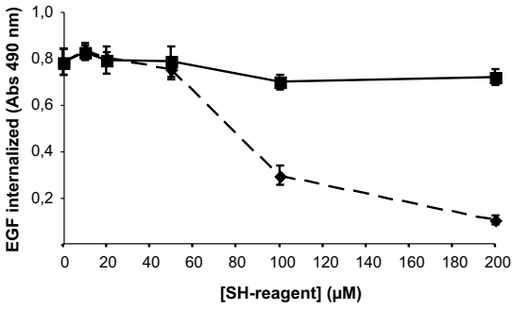


Fig. 5. Effect of SH reagents NEM and DTNB on EGF receptor internalization. HER14 cells were grown in 96-well plates and after washing with PBS, cells were incubated with EGF-biotin (50 ng/ml) in the absence or presence of 10, 20, 50, 100 or 200 µM of NEM (◆) or DTNB (■) in PBS_{gluc} for 20 min at 37°C. Then, cells were washed twice with PBS at 0°C, followed by treatment with acid wash. After fixation and permeabilization of the cells, the amount of internalized EGF-biotin was determined as described in *Materials and Methods*. Results +/- SEM of a representative experiment are shown (*n*=4).

To ascertain that the binding of EGF-biotin to the EGF receptor was not affected by NEM, cells were treated with EGF-biotin in the absence or presence of increasing concentrations of NEM for 60 min at 0°C. Subsequently, the amount of bound EGF-biotin was determined as previously described.[20] This revealed that NEM did not have an effect on the binding of EGF-biotin to the EGF receptor (Table 1), indicating that this was not the cause of the inhibition of EGF receptor internalization by NEM. Furthermore, lactate dehydrogenase (LDH) release was negligible after treatment of cells with increasing concentrations of NEM for 20 min at 37°C, showing that the decrease in internalized EGF in the presence of NEM was not due to a loss of cell integrity (Table 1).

Table 1. Effect of increasing concentrations of NEM on the binding of EGF after treatment of HER14 cells for 60 min at 0°C and on LDH release after treatment for 20 min at 37°C.

	Average ± SD (n=4)	
[NEM] (μM)	*Binding (Abs 490 nm)*	*LDH release (%)*
0	1.91 ± 0.10	3.00 ± 0.19
10	1.91 ± 0.07	3.35 ± 0.53
20	1.98 ± 0.06	2.87 ± 0.23
50	2.00 ± 0.05	2.66 ± 0.36
100	1.94 ± 0.06	2.35 ± 0.27
200	1.95 ± 0.08	2.46 ± 0.39

Effect of H_2O_2 and recovery on the cellular ratio of GSSG:GSH

A major cellular compound that regulates the cellular redox (and SH) status is glutathione and thus, the effect of treatment of cells with H_2O_2 on glutathione was investigated. Because both EGF receptor internalization and ubiquitination were inhibited by H_2O_2 already after 10 min of incubation (Fig. 1),[19] the effect of H_2O_2 on the cellular ratio of GSSG:GSH was determined at this time point as well. Therefore, HER14 cells were treated with 1, 2 or 5 mM of H_2O_2 for 10 min at 37°C, and the ratio GSSG:GSH was subsequently determined as described in *Materials and Methods*. As shown in Figure 6, treatment of cells with increasing concentrations of H_2O_2 resulted in a dose-dependent increase in the ratio of GSSG:GSH, resulting from a decrease in GSH and a coincident increase in GSSG (not shown). This increase in the cellular ratio of GSSG:GSH correlated with a concentration-dependent decrease in EGF receptor internalization after 10 min of incubation, as shown in Figure 6.

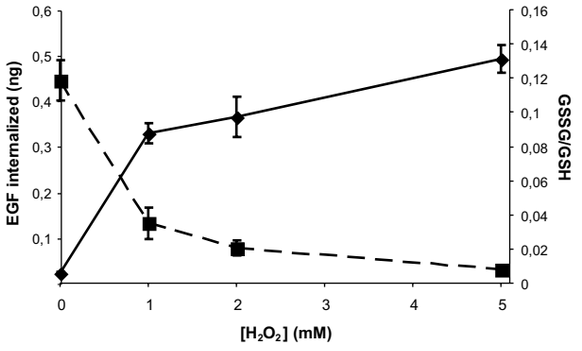


Fig. 6. Effect of increasing concentrations of H_2O_2 on the cellular ratio of GSSG:GSH and EGF receptor internalization. To determine the ratio of GSSG:GSH (◆), HER14 cells were washed with PBS and incubated with 0, 1, 2 or 5 mM H_2O_2 in PBS_{gluc} for 10 min at 37°C. Then, the cellular ratio of GSSG:GSH was determined as described in *Materials and Methods*. Results +/- SEM of a representative experiment out of three independent experiments are shown (*n*=3). To determine the effect of H_2O_2 on EGF receptor internalization (■), HER14 cells were washed with PBS and incubated with ^{125}I-EGF in the absence or presence of 1, 2 or 5 mM H_2O_2 for 10 min at 37°C. This was followed by treatment with acid wash and subsequently, the amount of internalized EGF was determined as described in *Materials and Methods*. Results +/- SEM of five independent experiments are shown.

To study the effect of recovery on the cellular levels of GSSG and GSH, HER14 cells were treated in the continuous presence of 5 mM H_2O_2 for 5, 10, 20, 30, 40 or 50 min. In addition, other cells were treated with 5 mM H_2O_2 for 10 or 20 min, followed by removal of the stress and recovery in PBS_{gluc} for 10, 20 or 30 min. This was again followed by determination of the cellular ratio of GSSG:GSH. Figure 7 shows that treatment of cells with 5 mM H_2O_2 for 5 min already induced a significant increase in GSSG:GSH levels as compared with control cells (no stress) with a maximum at 10 min of incubation. Thereafter, the cellular ratio of GSSG:GSH declined and reached a steady state upon 30 min of incubation. Removal of the stress both after 10 and 20 min of incubation resulted in a rapid recovery of the cellular ratio of GSSG:GSH almost to control levels within 10 min, leading to a complete restoration after recovery for 20 min (Fig. 7).

Discussion

In this study, we report the effect of H_2O_2 and H_2O_2 removal on EGF receptor internalization, ubiquitination and the cellular ratio of GSSG:GSH in fibroblasts. An inhibition of EGF receptor internalization and a coincident inhibition of mono-ubiquitination of Eps15 by H_2O_2 had been described previously.[19] Here, we show that the poly-ubiquitination of the EGF

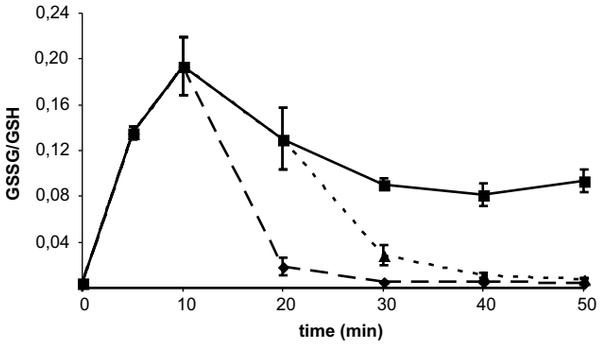


Fig. 7. Effect of H_2O_2 and H_2O_2 removal on the cellular ratio of GSSG:GSH. HER14 cells were washed with PBS and continuously incubated with 5 mM H_2O_2 for 5, 10, 20, 30, 40 or 50 min (■). Other cells were incubated with 5 mM H_2O_2 for 10 (◆) and 20 (▲) min, followed by removal of the stress and further incubation in PBS_{gluc} as described in *Materials and Methods*. Results +/- SEM of a representative experiment out of three independent experiments are shown (*n*=3).

receptor was inhibited in the presence of H_2O_2 under the same conditions that blocked EGF receptor internalization.

We discussed earlier that, although H_2O_2 inhibited EGF receptor internalization, pretreatment of cells with H_2O_2 was not sufficient to accomplish this inhibition.[19] Therefore, it was suggested previously that the interference of H_2O_2 with the EGF receptor-mediated endocytosis was rapidly reversible. Here, we do indeed show that EGF receptor internalization was reversibly inhibited by H_2O_2 and that complete re-establishment of internalization after removal of 5 mM H_2O_2 required a recovery time of at least 30 min. In the recovery experiments, H_2O_2 was not removed by the addition of catalase, but both H_2O_2 and EGF were removed by washing the cells, followed by further incubation in PBS_{gluc}. This revealed that EGF-induced receptor internalization upon recovery was due to activation of those receptors that had bound EGF during the pre-incubation in the presence of H_2O_2 and not to the binding of EGF to another pool of receptors after H_2O_2 removal. Therefore, we can conclude with certainty that the inhibitory effect of H_2O_2 on EGF receptor internalization was reversible.

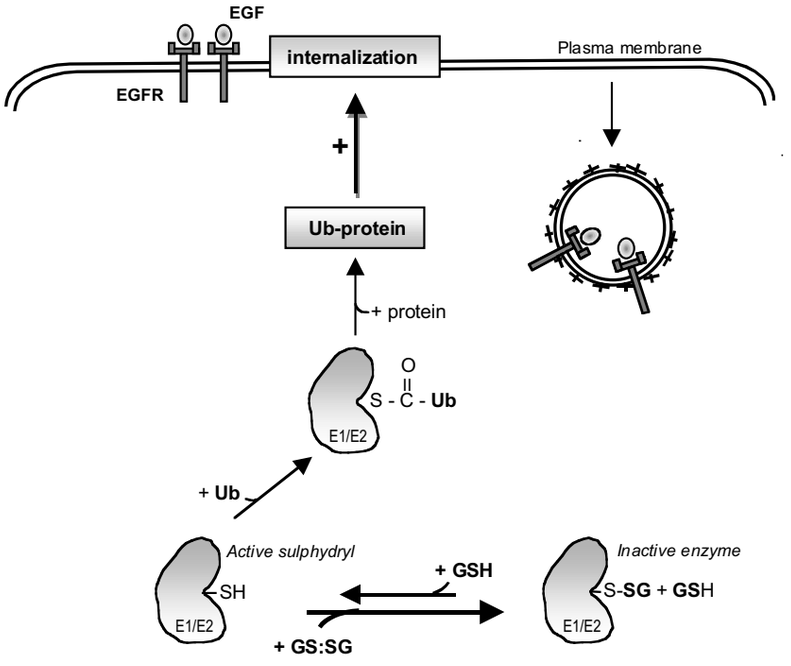
Although the precise role of ubiquitination in the endocytosis of the EGF receptor has not been elucidated, ubiquitin-dependent internalization of other receptors has been described. Mutation of the ubiquitination sites on the α-factor receptor in yeast abolished receptor endocytosis and, in addition, it was shown that mono-ubiquitination of the α-factor receptor was sufficient to trigger its internalization.[24,41] Other studies in yeast cells revealed that ubiquitin itself probably functions as an internalization signal and triggers downregulation

after its linkage to a plasma membrane protein.[42] A role for ubiquitination has also been demonstrated for cell surface receptors in mammalian cells. The internalization of the growth hormone receptor is dependent on the ubiquitin conjugation system [25,26] and multi-ubiquitination of the colony-stimulating factor-1 receptor at the plasma membrane is followed by its internalization.[43] Recent studies revealed that both the mono-ubiquitination of Eps15 and the poly-ubiquitination of the EGF receptor most likely occur at the plasma membrane,[23] suggesting a role for ubiquitin in EGF receptor-mediated endocytosis. In this study, we showed that both the mono-ubiquitination of Eps15 and the poly-ubiquitination of the EGF receptor were inhibited by H_2O_2 and that inhibition of ubiquitination was accompanied by an inhibition of EGF receptor internalization. Therefore, these results suggest that the internalization of the EGF receptor might also be ubiquitin-dependent.

A rapid and dose-dependent loss of endogenous ubiquitin-protein conjugates has been observed upon exposure of bovine retina cells or bovine lens epithelial cells to H_2O_2 and was consistent with reductions in ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) activities.[27,28] Furthermore, decreased E1 and E2 activities were inversely related with the cellular ratio of GSSG:GSH. Therefore, it was proposed that increases in the cellular ratio of GSSG:GSH resulted in a rapid S-thiolation of E1 and E2 active-site sulphydryls by GSSG.[28,29] In the present study, we showed that treatment of cells with the membrane permeable thiol blocking agent NEM resulted in a decrease in the amount of internalized EGF, whereas treatment with the membrane-impermeable SH reagent DTNB had no effect (Fig. 5). Furthermore, the poly-ubiquitination of the EGF receptor was inhibited by NEM under the same conditions that inhibited EGF receptor internalization (not shown). Therefore, this indicates that intact intracellular SH groups are required for both ubiquitination and EGF receptor internalization. In addition, we found a dose-dependent increase in the ratio of GSSG:GSH after treatment of HER14 cells with H_2O_2 (Fig. 6). An increase in GSSG:GSH levels was already observed after treatment of cells with H_2O_2 for 5 min (Fig. 7), followed by a maximum at 10 min. This rapid increase was due to an increase in absolute amounts of GSSG and a coincident decrease in the concentration of GSH (not shown), probably due to glutathione peroxidase activity. The dose-dependent increase in the GSSG:GSH ratio correlated with a dose-dependent inhibition of EGF receptor internalization (Fig. 6) and coincided with an inhibition of ubiquitination of Eps15 [19] and the EGF receptor (Fig. 1). Therefore, these data suggest a causal relation between inhibition of EGF receptor internalization, ubiquitination and increased ratio of GSSG:GSH.

Thiolation of the sulphydryl group of ubiquitination enzymes was shown to be reversible, and studies performed with retinal pigment epithelial cells revealed that both ubiquitin-conjugating activity and increased GSSG:GSH levels re-established to control levels upon removal of H_2O_2.[29] We showed that the increased ratio of GSSG:GSH re-established within 10 min upon removal of H_2O_2 and restored to control levels (no stress)

after 20 min of recovery (Fig. 7). In addition, it was shown that both the inhibition of ubiquitination of the EGF receptor and of Eps15 also recovered upon stress removal and reached control levels approximately after 20-30 min (Figs. 3 and 4). This indicates that both ubiquitination and cellular ratio of GSSG:GSH were reversibly affected by H_2O_2 and revealed that GSSG:GSH levels restored prior to recovery of the ubiquitination. Furthermore, because complete restoration of internalization after removal of 5 mM H_2O_2 required a recovery time of at least 30 min (Fig. 2), these data imply that recovery of the cellular ratio of GSSG:GSH



During oxidative stress

Fig. 8. Schematic model of the hypothesized mechanism underlying inhibition of EGF receptor internalization by H_2O_2. In the absence of oxidative stress, both ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) are able to bind ubiquitin (Ub) via an active sulphydryl (SH) group, leading to the activation and transfer of ubiquitin to a substrate protein (Ub-protein). Although the exact role of ubiquitin has not been established, ubiquitination might be required for EGF receptor-mediated endocytosis. During oxidative stress, induced by exposure of cells to H_2O_2, the cellular GSSG:GSH ratio will be increased. Subsequently, GSSG reversibly induces thiolation of the active sulphydryl of E1 and E2, leading to the inactivation of these enzymes. As a result, ubiquitination of proteins that are required for EGF receptor internalization is inhibited in the presence of H_2O_2, resulting in an inhibition of EGF receptor internalization. Removal of H_2O_2 results in recovery of the GSSG:GSH ratio, followed by dethiolation of the ubiquitination enzymes and restoration of their activities.

might be required for re-establishment of ubiquitination and of subsequent EGF receptor internalization. Moreover, preliminary results revealed that decreasing total glutathione content by treatment of cells with buthionine sulfoximine, resulted in an increased sensitivity of cells to H_2O_2 related to EGF receptor internalization (results not shown). Because lowered total glutathione content might result in a more rapid increase in GSSG:GSH at low concentrations of H_2O_2, the data are in line with the hypothesis that EGF receptor internalization is regulated by the cellular GSSG:GSH ratio. Therefore, we conclude that the results shown in this study further support the hypothesis that the internalization of the EGF receptor might be inhibited by an inhibition of ubiquitination of proteins that are involved in EGF receptor-mediated endocytosis, probably regulated by the cellular ratio of GSSG:GSH. A schematic model is drawn in Figure 8.

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Chapter 6

Summarizing Discussion

Development of cellular large-scale screening assays

This thesis described the effects of exposure of cells to oxidative stress, induced by H_2O_2, on the functioning of proteins involved in signal transduction pathways. In addition, H_2O_2 was chosen as oxidant in order to produce cellular screening assays to measure antioxidant efficacy in preventing the H_2O_2-induced modifications of protein functioning.

A family of kinases that plays a key role in the transduction of extracellular signals into intracellular events is formed by the MAP kinases.[1] Moreover, MAP kinases are rapidly activated in response to various extracellular signals, including different types of cellular stress.[2-5] Therefore, the effect of H_2O_2 on the phosphorylation of MAP kinase was investigated and this revealed that exposure of Rat-1 fibroblasts resulted in a transient phosphorylation of p44/p42^{MAPK}. Subsequently, the H_2O_2-induced phosphorylation of p44/p42^{MAPK} was used as a marker for oxidative stress and the availability of a phospho-specific p44/p42^{MAPK} antibody provided us the ability to develop a cellular enzyme-linked immunosorbent assay (Cell-ELISA) to measure the phosphorylation of p44/p42^{MAPK} (*chapter 2*). This assay was subsequently used for the screening of antioxidant efficacy in Rat-1 fibroblasts and in addition, the assay was applicable to test other stresses, such as menadione, cumene hydroperoxide, AMVN and hypoxanthine/xanthine oxidase.

A second screening assay was developed to measure the internalization of the EGF receptor in 96-well plates (*chapter 4*). Internalization and subsequent degradation of activated EGF receptors, also referred to as receptor downregulation or receptor-mediated endocytosis, results in a reduction of the amount of EGF receptors expressed at the plasma membrane and therefore in a reduction of binding sites for EGF. Another cellular feedback mechanism to attenuate receptor signaling involves the activation of phosphatases.[6] Dephosphorylation of the C-terminal Tyr residues of the EGF receptor, for instance, abrogates docking sites for downstream signaling proteins and furthermore, the enzymatic activity of several signaling proteins can be negatively regulated by dephosphorylation. Finally, a third mechanism to regulate EGF-induced signaling is receptor transmodulation, which results in lowered affinity of the receptor for its ligand and in addition, receptor Tyr kinase activity is reduced.[7] We decided to investigate the effect of H_2O_2 on receptor downregulation, because an inhibition of EGF receptor-mediated endocytosis had been described for different forms of cellular stress,[8] suggesting that oxidative stress might interfere with this process as well. H_2O_2 was found to inhibit the internalization of the EGF receptor in HER14 fibroblasts (*chapter 3*) and this inhibition was subsequently considered as a marker for oxidative stress. To easily study ligand-induced internalization, a cellular screening assay in 96-well plates was developed, which was partly based on the cellular MAP kinase assay as described in *chapter 2*. In this assay, internalization was studied using biotin-conjugated EGF and we showed that the results obtained with this internalization assay were comparable with results obtained with radioactive labeled EGF (*chapter 4*).

In conclusion, the newly developed 96-well plate assays are nonradioactive, relatively fast and reliable methods for quantitative detection of changes in phosphorylation of MAP kinases or changes in ligand-induced internalization in 96-well plates. Furthermore, both assays are applicable for the screening of various stress conditions on these processes and for testing a variety of antioxidants in their capacity to prevent or reduce the H_2O_2-induced changes in these cellular responses.

Regulation of EGF receptor internalization by the cellular redox status

The studies as described in *chapters 3* and *5* of this thesis focused on the interference of H_2O_2 with the downregulation of the EGF receptor. These studies suggest a relation between EGF receptor internalization, the ubiquitin conjugation system and the cellular redox status and we have proposed that H_2O_2 inhibits EGF receptor internalization via inhibition of ubiquitination, regulated by the cellular redox status (*chapter 5*, Fig. 8). This is based on the fact that treatment of cells with H_2O_2 resulted in a rapid increase in the cellular ratio of GSSG:GSH (*chapter 5*, Fig. 7), due to an increase in absolute levels of GSSG and a coincident decrease in GSH. This rapid increase in GSSG:GSH levels is probably due to glutathione peroxidase activity, which converts H_2O_2 at the expense of GSH, thereby forming GSSG. There was a maximum in the GSSG:GSH ratio after 10 min of H_2O_2 exposure, followed by a decrease. This decrease can be explained by glutathione reductase activity that, in a reaction requiring NADPH, catalyzes reduction of GSSG to replenish GSH. In addition, a decrease in GSSG:GSH might be due to reaction of GSSG with SH groups of proteins, resulting in a decrease in GSSG and a coincident increase in GSH. Conjugation of GSSG to essential SH groups of enzymes would result in a reduction or inhibition of their enzymatic activity, as proposed for the ubiquitination enzymes (*chapter 5*, Fig. 8).

The same mechanism has recently been postulated to result in inactivation of phosphatases by H_2O_2.[9] As described in *chapter 1*, the PTPs that are attacked by ROS all share the same catalytic site Cys. Under physiologic conditions, most Cys in cytosolic proteins are in the protonated (SH) form. One of the mechanisms for transient inactivation of the catalytic site Cys in phosphatases by ROS might occur directly through oxidation of the Cys to form a sulfenic acid (Fig. 1).[10] In the sulfenic form, the enzymatic or catalytic activity of the phosphatase is significantly reduced or even abolished. Continued high levels of oxidative stress might lead to an irreversible oxidation of the Cys to a sulfinic ion.[11] H_2O_2-induced reversible inactivation of phosphatases might, however, also occur indirectly via formation of a mixed glutathione intermediate (Fig. 1).[9] This is confirmed by the observation that stimulation of A431 cells with EGF induces transient glutathionylation of the active site Cys of PTP-1B.[12] The glutathionylated PTP can subsequently be regenerated into the active form probably by further reduction by GSH in a reaction catalyzed by thioredoxin.[9,11]

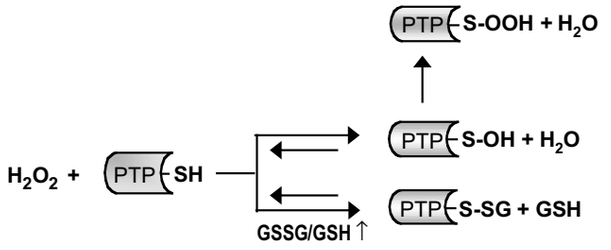

Fig. 1. Postulated mechanism of inactivation of phosphatases by H_2O_2 (adapted from reference 9). H_2O_2 causes reversible inactivation of protein tyrosine phosphatases (PTPs) via oxidation of the essential SH group within their active site Cys. This may occur directly through formation of a sulfenic acid intermediate (PTP-SOH) or indirectly via formation of a mixed glutathione intermediate (PTP-S-SG). The oxidized, inactive phosphatase can be regenerated into the active form by reduction by GSH in a reaction catalyzed by thioredoxin. Continued high levels of oxidant stress might lead to a most likely irreversible oxidation to a sulfinic ion (PTP-SOOH).

Reversible inactivation of ubiquitination enzymes E1 and E2 by transient glutathionylation is supported by other studies that revealed that addition of GSSG to supernatants of retina cells resulted in diminished formation of E1- and E2-^{125}I-labeled ubiquitin thiol esters and coincident reductions in protein-^{125}I-labeled ubiquitin conjugates.[13] In addition, restoration of GSSG:GSH ratios in supernatants of H_2O_2-treated retinas by the addition of GSH resulted in a partial restoration of E1 and E2 activities and protein-^{125}I-labeled ubiquitin conjugates. This indicates that ubiquitination enzyme activities are dependent on the cellular GSSG:GSH ratio and suggests that inactivation of ubiquitination enzymes might be regulated by reversible glutathionylation. To confirm that ubiquitination enzyme activities of E1 and E2 were thiol-dependent, cells were treated with the thiol-specific oxidant diamide.[14] Diamide preferentially oxidizes low molecular weight thiols as opposed to protein thiols and treatment of cells with diamide results in the oxidation of the nonprotein thiol GSH to GSSG. These studies revealed that treatment of retinal epithelial cells with diamide resulted in a dose-dependent increase in the cellular GSSG:GSH ratio which was accompanied by a dose-dependent decrease in ubiquitin-protein conjugates and reductions in E1 and E2 activities.[14] Therefore, the results of these studies support the hypothesis that transient inactivation of ubiquitination enzyme activities is regulated by the cellular thiol redox status. In line with these results and with the model as proposed in *chapter 5* (Fig. 8), we observed in preliminary studies a concentration-dependent inhibition of EGF receptor internalization in diamide-treated HER14 cells (results not shown). Furthermore, preliminary experiments revealed that decreasing total glutathione content by treatment of cells with buthionine sulfoximine (BSO) resulted in an increased sensitivity of cells to H_2O_2 at the level of EGF receptor internalization (results not shown). Lowered total glutathione content might

result in increased sensitivity due to a more rapid increase in GSSG:GSH at low concentrations of H_2O_2. Therefore, this further supports the model that regulation of EGF receptor internalization is indeed dependent on the cellular redox status.

Role of ubiquitin conjugation system in receptor-mediated endocytosis

Until now, the exact role of the ubiquitination system in EGF receptor internalization has not been revealed and it remains unanswered whether poly-ubiquitination of the EGF receptor itself or ubiquitination of other proteins is involved. Another transmembrane receptor that undergoes poly-ubiquitination in response to ligand-stimulation is the Growth Hormone Receptor (GHR).[15,16] Furthermore, the internalization of the GHR has been shown to be dependent on the ubiquitination system.[16] The cytoplasmic tail of the GHR carries a ubiquitin-dependent endocytosis motif and GHR internalization seems to require the recruitment of the ubiquitin conjugation system to this motif rather than ubiquitination of the GHR itself.[17] One of the models postulated for ligand-induced GHR internalization includes the recruitment of the ubiquitin conjugation system and the coincident binding of a regulatory protein to the GHR.[17] This is followed by ubiquitination of the regulatory protein, which is subsequently recognized by an endocytic adaptor protein, leading to GHR internalization. A potential candidate that may function as a regulatory protein to mediate GHR internalization has been proposed to be Eps15.[17]

Based on the findings obtained for the GHR, EGF receptor internalization is possibly not dependent on poly-ubiquitination of the EGF receptor itself. Poly-ubiquitination of proteins generally serves as a recognition signal for destruction by the proteasome. In line with this, it has become evident that poly-ubiquitination of the EGF receptor probably targets receptors into the degradative pathway in which proteasomal and lysosomal hydrolyses may respectively degrade the cytoplasmic and exoplasmic domains of the receptor.[18,19] Furthermore, regulation of EGF receptor internalization might be comparable with GHR internalization. The cytoplasmic tail of the EGF receptor might be responsible for recruitment of the ubiquitin conjugation system, followed by ubiquitination of both the receptor and a regulatory protein that is recruited to the receptor and plays an essential role in EGF receptor internalization. An excellent candidate would indeed be the EGF receptor pathway substrate Eps15, which is recruited to the plasma membrane upon EGF stimulation, then localizes at the rim of clathrin-coated pits and binds to the adaptor protein AP-2.[20-22] Moreover, recent studies using site-directed mutagenesis showed that Tyr phosphorylation of Eps15 is required for ligand-induced internalization of the EGF receptor.[23] The question whether mono-ubiquitination of Eps15 is also a prerequisite for EGF receptor internalization remains to be resolved.

An enzyme that has been found to be involved in ubiquitination of the EGF receptor is the 120 kDa protein c-Cbl.[24] The N-terminal half of c-Cbl contains a Tyr kinase binding

domain and upon EGF stimulation c-Cbl binds to the EGF receptor and undergoes Tyr phosphorylation. The C-terminal sequences of c-Cbl comprises several structural units including a conserved RING finger,[25] which has been proposed to be involved in regulation of protein ubiquitination.[24] In *vitro* studies revealed that c-Cbl functions as a component of the ubiquitin ligation (E3) machinery and that c-Cbl adaptor proteins recruit ubiquitin-activating (E1) and -conjugating (E2) enzymes.[18] In addition, the RING finger of c-Cbl might also be involved in recruitment of the E2 enzyme.[18] It has been demonstrated that the most N-terminal Cys in the RING finger of c-Cbl is essential for its functioning and furthermore, treatment of c-Cbl with NEM completely abolished its activity.[18] These results therefore imply that not only ubiquitination enzymes E1 and E2 might become reversibly inactivated via oxidation of essential SH groups within their active site Cys. Moreover, treatment of cells with H_2O_2 might result in an inhibition of ubiquitin ligase (E3) activity as well via oxidation of the essential Cys localized in its RING finger.

Inhibition of receptor-mediated endocytosis by hydrogen peroxide in general

CSLM studies revealed that H_2O_2 not only affected the internalization of the EGF receptor, but also receptor-mediated endocytosis of the Chicken Hepatic Lectin (CHL) receptor (*chapter 3*, Fig. 5). This suggests that receptor-mediated endocytosis might be inhibited in general by H_2O_2. On the other hand, the model as proposed in *chapter 5* (Fig. 8) suggests that the uptake of receptors that are internalized in a ubiquitin-independent manner may not be affected. Internalization of the transferrin receptor (TfR) was shown to proceed normally in cells that exhibit a temperature-sensitive defect in their ubiquitin conjugation system.[16] Therefore, internalization of the TfR is considered to be ubiquitin-independent. Upon ligand binding, the TfR is internalized via clathrin-coated pits and then recycles between the cell surface and endosomes, thereby providing cells with iron. It has been described that treatment of human hematopoietic cell lines with H_2O_2 results in a rapid and marked reduction of the amount of plasma membrane TfR.[26] This H_2O_2-induced downregulation of TfRs from the cell surface was not due to receptor loss, but to redistribution of the TfR.[26] Therefore, this suggests that the TfR was normally internalized, but did not recycle to the cell surface. This H_2O_2-induced inhibition of TfR recycling also indicates that receptor-mediated endocytosis can be inhibited at different stages by H_2O_2, as also discussed for the EGF receptor in *chapter 3*. There is strong evidence that H_2O_2, next to internalization, also inhibits the degradation of internalized EGF. Even when ^{125}I-EGF was internalized, its degradation was found to be inhibited in the presence of H_2O_2, because no free ^{125}I was detected in the incubation medium after incubation of HER14 cells for 30 or 60 min at 37°C (*chapter 3*, Fig. 4 and data not shown). Therefore, these results indicate that receptor-mediated endocytosis is also inhibited al later stages than internalization, possibly due to inhibition of intravesicular trafficking.

In conclusion, receptor-mediated endocytosis might be inhibited by oxidative stress at different stages. Moreover, because ubiquitin-independent internalization of the TfR was not affected, these data are in agreement with our model as postulated in *chapter 5* (Fig. 8), thereby assuming that the ubiquitin conjugation system is the only target of H_2O_2 that is responsible for inhibition of EGF receptor internalization.

Effects of inhibition of EGF receptor internalization by oxidative stress on cellular functioning

An important issue concerns the consequence of inhibition of EGF receptor internalization by oxidative stress on cellular functioning. EGF receptor downregulation has always been considered as one of the negative feedback mechanisms to attenuate EGF-induced receptor signaling. Therefore, inhibition of EGF receptor internalization might result in enhanced and prolonged receptor signaling and therefore in sustained phosphorylation and activation of signaling proteins involved in cellular responses such as proliferation and differentiation. On the other hand, it becomes more and more apparent that the EGF receptor must still be active in the early endosome where it can still phosphorylate and activate substrate proteins.[27-30] This suggests that inhibition of internalization during oxidative stress prevents signaling from the endosome compartment. It has been demonstrated that the inability of cells to undergo receptor-mediated endocytosis can result in cellular transformation or tumor formation.[31,32] Therefore, long exposure of cells to oxidative stress, for instance during chronic inflammation, might result in uncontrolled cell proliferation. However, ROS have numerous biomolecular targets, which might have counteracting effects. Severe DNA damage, for instance, might result in a cell cycle arrest and even in programmed cell death. Therefore, it is difficult to predict the result on cellular functioning upon inhibition of EGF receptor internalization and the final outcome is dependent on processes that overrule the others.

H_2O_2 – a second messenger in signal transduction

Initially, we thought that H_2O_2 was a rather artificial oxidative stress inducer. At present, it is known that H_2O_2 can act as second messenger in signal transduction. Therefore, the effects we discuss are highly relevant for understanding the possible role of H_2O_2 as second messenger. Furthermore, our results contribute to the understanding of the mechanism of EGF receptor internalization and its regulation.

As described in *chapter 1*, stimulation of cells with EGF induces the intracellular production of H_2O_2,[33] that then acts as second messenger. The EGF-induced H_2O_2-production is required for EGF receptor phosphorylation, which is most likely accomplished via reversible inactivation of PTPs.[34] This EGF-induced generation of H_2O_2 might, however, also play a physiologic role in EGF receptor-mediated endocytosis. Upon EGF stimulation,

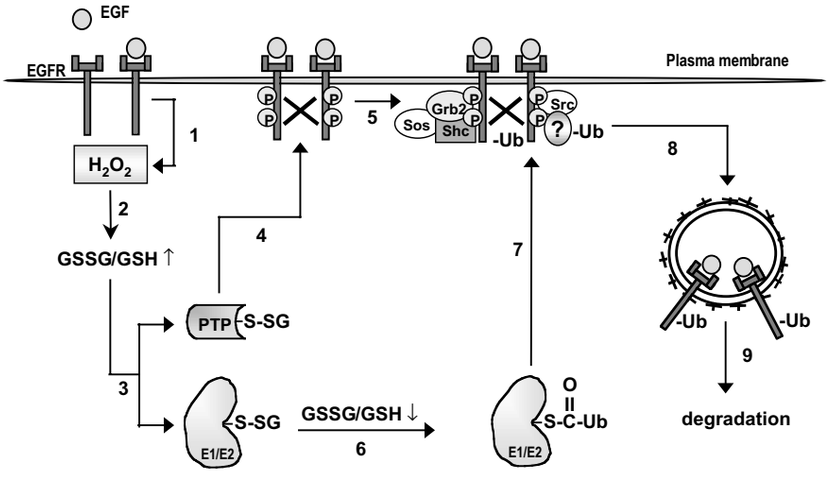

Fig. 2. Postulated model for the role of H_2O_2 as second messenger in EGF-induced signal transduction. Stimulation of cells induces intracellular H_2O_2-production (1). Due to glutathione peroxidase activity, which converts H_2O_2 at the expense of GSH, the cellular ratio of GSSG:GSH will increase (2). GSSG then reacts with the active SH groups of PTPs and ubiquitination enzymes E1 and E2, leading to inactivation of their enzymatic activity (3). Transient inactivation of PTPs is required for phosphorylation of the EGF receptor (4) and coincident transient inactivation of ubiquitination enzymes results in a delay of EGF receptor internalization. This allows the recruitment of downstream signaling proteins, such as Shc, Grb2 and Sos to the receptor, followed by their activation (5). In addition, the receptor becomes Tyr phosphorylated on heterologous phosphorylation sites, for example by pp60c-src (src). Then, as a result of glutathione reductase activity, the cellular GSSG:GSH ratio will decrease (6), resulting in reduction of the glutathionylated SH group of ubiquitination enzymes. Thereby, the ubiquitination enzymes become activated and can subsequently transfer ubiquitin (Ub) (7) to both the EGF receptor and an unidentified regulatory protein, as indicated by a question mark. Ubiquitination of this regulatory protein subsequently induces EGF receptor internalization (8) and poly-ubiquitination of the EGF receptor targets the receptor into the degradative pathway (9), leading to transport of the EGF receptor to late endosomes and lysosomes, followed by degradation.

inhibition of the ubiquitination system by H_2O_2 might result in a delay of EGF receptor internalization, thereby allowing the EGF receptor to phosphorylate and activate substrate proteins at the plasma membrane. In Figure 2, the following model is suggested. Stimulation of cells with EGF leads to intracellular H_2O_2-production [33] (1) and a subsequent increase in the cellular GSSG:GSH ratio (2). GSSG then reversibly reacts with essential SH groups of PTPs [9] (3), leading to a transient reduction of their enzymatic activity, which is required for cross- or autophosphorylation of the EGF receptor [33,34] (4). Then, because of reversible glutathionylation of ubiquitination enzymes by H_2O_2 (3), the internalization of the EGF receptor is delayed and downstream signaling proteins are recruited to the plasma

membrane to become phosphorylated and activated by the EGF receptor (5). Due to glutathione reductase activity, the cellular GSSG:GSH ratio then decreases (6), followed by reduction of essential SH groups and activation of PTPs and ubiquitination enzymes. Subsequently, both the EGF receptor and a regulatory protein become ubiquitinated (7), followed by EGF receptor internalization (8). Poly-ubiquitination of the EGF receptor then targets the receptor into the degradative pathway (9), including transport of the EGF receptor to the late endosomes and lysosomes, followed by degradation.

One of the important issues that still is a point of debate is the site of intracellular H_2O_2-production. In phagocytic cells, ROS are produced through the assembly of NADPH oxidase, which is composed of both membrane-bound proteins and cytosolic factors.[35] One of the components involved in NADPH oxidase assembly is rac2, a small GTPase that is primarily expressed in phagocytic cells. Its homologue rac1, however, is expressed in fibroblasts and other non-phagocytic cells. Activation of mutants of rac1 has been shown to stimulate ROS production in fibroblasts,[36-40] suggesting that rac1 might be involved in ligand-induced ROS production. Furthermore, this suggests that non-phagocytic cells may have a molecular oxidase complex comparable with the NADPH oxidase of phagocytic cells.[11] This is confirmed by others that claim that plasma membrane redox systems that would generate ROS such as superoxide and H_2O_2 are expressed at the plasma membrane in every kind of cell.[41]

Another important question that remains unanswered in this model is where dephosphorylation of the EGF receptor occurs. The fact that several studies showed the presence of active receptors in the early endosomes [27-30] suggests that EGF receptor dephosphorylation occurs after internalization. Therefore, reduction and activation of phosphatases might also occur after uptake of the EGF receptor by the cell. On the other hand, the requirement of reducing conditions to activate the ubiquitination sytem prior to receptor internalization suggests that phosphatases might be reduced and thus activated at the plasma membrane. This, in turn, might lead to dephosphorylation of the EGF receptor prior to internalization. Therefore, we suggest that restoration of PTP activity possibly results in partial dephosphorylation of the EGF receptor at the plasma membrane, whereas activation of different pools of PTPs might be involved in complete dephosphorylation after receptor internalization, finally leading to attenuation of receptor signaling.

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Nederlandse Samenvatting

Samenvatting

Onze atmosfeer bestaat voor 21% uit zuurstof en dit maakt het voor organismen die zuurstof gebruiken (aërobe levensvormen) mogelijk om te overleven. Zo consumeert het menselijk lichaam zuurstof en vele cruciale lichaamsfuncties kunnen zelfs niet langer dan een paar minuten zonder zuurstof overleven. Zuurstof die we inademen wordt in het bloed opgenomen en vervolgens via het bloed naar alle organen vervoerd. Organen zijn opgebouwd uit weefsel en weefsels bestaan op hun beurt uit cellen. Cellen zijn de kleinste functionele eenheden van een weefsel die de eigenschappen van dat weefsel bezitten. Vanuit het bloed wordt zuurstof in de lichaamscellen opgenomen en in de cel wordt zuurstof vervolgens omgezet tot water, waarbij energie vrijkomt. Bij deze omzettingen van zuurstof naar water worden er echter ook reactieve tussenproducten gevormd, die we vrije *zuurstofradicalen* noemen. Radicalen zijn moleculen met een of meerdere ongepaarde electronen (negatief geladen deeltjes) en door deze ongepaarde electronen zijn radicalen erg reactief. Door reactie van radicalen met andere moleculen (oxidatie) kunnen er nieuwe radicalen worden gevormd en op deze manier kunnen er kettingreacties ontstaan van radicaalproductie. In de cel kunnen zuurstofradicalen reageren met (en daarmee schade aanrichten aan) DNA (genetisch materiaal), lipiden (vetten) en eiwitten, wat grote gevolgen kan hebben voor het functioneren van de cel.

Zuurstofradicalen worden niet alleen tijdens normaal (aëroob) celmetabolisme gevormd, maar ook tijdens ontstekingsreacties. Ontstekingscellen maken zuurstofradicalen die ze gebruiken om indringers, zoals bacteriën, te doden. Door lekkage van zuurstofradicalen uit de ontstekingscellen worden omliggende cellen blootgesteld aan deze radicalen en kunnen hierdoor worden beschadigd. Daarnaast zijn er ook een aantal omgevingsfactoren, zoals ultraviolet licht en sigarettenrook, die in lichaamscellen de productie van radicalen verhogen.

Als cellen worden blootgesteld aan een verhoogde concentratie van zuurstofradicalen dan noemen we dit *oxidatieve stress*. Ter bescherming tegen zuurstofradicalen bevatten cellen een aantal afweersystemen, die we *antioxidante afweermechanismen* noemen. Zo heeft elke cel een aantal antioxidante enzymen, die de reactieve zuurstofradicalen omzetten in niet-reactieve stoffen. Daarnaast zijn er antioxidanten of vitaminen die we voornamelijk via ons voedsel binnen krijgen en die de cel beschermen tegen schadelijke zuurstofradicalen. Onder normale omstandigheden is er in de cel een balans tussen enerzijds de productie van radicalen en anderzijds de antioxidante mechanismen. Echter, als deze balans wordt verstoord, bijvoorbeeld door een overproductie van radicalen of door het niet optimaal functioneren van de antioxidante afweermechanismen, kan dit leiden tot oxidatieve stress. De laatste jaren is uit onderzoek gebleken dat oxidatieve stress een rol speelt in een aantal ziektes, zoals atherosclerosis

(vaatvernauwing door aderverkalking), chronische ontstekingsreacties, kanker, neurologische degeneratieve processen zoals Alzheimer en in verouderingsprocessen. Daarom is het van belang te onderzoeken wat de effecten van oxidatieve stress op de cel zijn en of we deze effecten, bijvoorbeeld door het toedienen van antioxidanten, kunnen tegengaan.

In dit proefschrift zijn een aantal effecten van oxidatieve stress op het niveau van eiwitten beschreven. Daarnaast zijn er twee methoden *(assays)* ontwikkeld die gebruikt kunnen worden om de beschermende werking van antioxidanten te kunnen testen. Er is vooral gekeken naar de effecten van oxidatieve stress op eiwitten die betrokken zijn bij *signaaltransductie*. Signaaltransductie is de overdracht van een signaal van buiten de cel naar binnenin de cel. Deze signaaloverdracht is van groot belang voor de onderlinge communicatie tussen cellen en kan bijvoorbeeld plaatsvinden door middel van *signaalstoffen* die door de ene cel worden geproduceerd en uitgescheiden en door andere cellen als het ware worden ontvangen. Voorbeelden van zulke signaalstoffen zijn *hormonen* en *groeifactoren* die door gespecialiseerde cellen worden gemaakt en uitgescheiden en zich vervolgens in het bloed en tussen de cellen bevinden. Vervolgens kunnen de signaalstoffen aan specifieke cellulaire eiwitten binden, die we *receptoren* noemen. Deze receptoren steken dwars door het omhulsel van de cel, de celmembraan, heen en door binding van signaalstoffen worden de receptoren geactiveerd en geven op deze manier het signaal door van de buitenkant van de cel naar de binnenkant. Vervolgens wordt het signaal in de cel via allerlei interacties van eiwitten doorgegeven naar de celkern, waar zich het DNA bevindt. Afhankelijk van het signaal dat van buitenaf gegeven was worden er bepaalde genen geactiveerd en dit leidt uiteindelijk tot een cellulaire respons, zoals proliferatie (celdeling) of differentiatie (specialisatie van de cel). Daarnaast kan de cel ook “besluiten” dat het beter is om dood te gaan en kan dan tot geprogrammeerde celdood (apoptose) overgaan. Dit laatste proces speelt bijvoorbeeld een belangrijke rol bij verouderingsprocessen.

Een van de eiwitten die een centrale rol speelt bij deze signaaltransductie of signaaloverdracht is *Mitogen-Activated Protein (MAP) kinase*. In **hoofdstuk 2** beschrijven we dat MAP kinase niet alleen wordt geactiveerd als cellen worden blootgesteld aan groeifactoren (signaalstoffen), maar ook als cellen worden blootgesteld aan oxidatieve stress. Deze oxidatieve stress wordt veroorzaakt doordat we van buitenaf *waterstofperoxide (H_2O_2)* aan de cellen hebben gegeven. Waterstofperoxide kan over de celmembraan diffunderen en binnen in de cel de productie van vrije zuurstofradicalen induceren, wat leidt tot oxidatieve stress. Deze activatie van MAP kinase tijdens oxidatieve stress hebben we daarna gebruikt om een assay te ontwikkelen waarin de activatie van MAP kinase door oxidatieve stress wordt gemeten in cellen. Deze assay is vervolgens toegepast om de beschermende werking van antioxidanten te meten, die van buitenaf aan de cellen werden toegevoegd.

Vervolgens zijn we gaan kijken naar effecten van oxidatieve stress op het functioneren van een bepaalde groeifactor receptor, de *epidermale groeifactor (EGF) receptor*. Onder normale omstandigheden leidt blootstelling van cellen aan EGF tot binding van EGF aan de EGF receptor, die zich in de celmembraan bevindt. Dit leidt tot activatie van de EGF receptor en tot signaaloverdracht van buiten naar binnen in de cel zoals hierboven is beschreven. Het is van groot belang dat de activatie van de EGF receptor goed wordt gereguleerd en dat de receptor op een gegeven moment weer wordt geïnactiveerd om de signaaloverdracht te stoppen. Eén van de manieren die de cel heeft om de signaaltransductie als het ware uit te doven is door de geactiveerde receptoren naar binnen te sluizen en vervolgens af te breken. Dit proces wordt ook wel *"receptor-gemedieerde endocytose"* genoemd en het naar binnen sluizen, wat dus een onderdeel is van dit endocytose proces, noemen we *"internalisatie"*. Door opname/internalisatie van receptoren in de cel neemt het aantal receptoren aan de celmembraan af en daarmee het aantal bindingsplaatsen voor EGF en dit resulteert in uitdoving van EGF receptor signalering. Deze receptor-gemedieerde endocytose is belangrijk, omdat bekend is dat een remming van dit proces tumorvorming tot gevolg kan hebben.

In **hoofdstuk 3** is beschreven dat de internalisatie van de EGF receptor wordt geremd tijdens oxidatieve stress (geïnduceerd door waterstofperoxide). Om hiervoor een verklaring te vinden hebben we vervolgens gekeken naar een aantal stappen die plaatsvinden vòòr receptor internalisatie. Uit deze studies bleek dat er tijdens oxidatieve stress wel normale binding van EGF aan de EGF receptor plaatsvindt en dat fosforylatie van de EGF receptor (een modificatie die normaal plaatsvindt na EGF stimulatie en die betrokken is bij het doorgeven van het signaal) niet is geremd. Ditzelfde geldt voor fosforylatie van Eps15, een eiwit dat na EGF stimulatie aan geactiveerde EGF receptoren bindt en een essentiële rol speelt bij de internalisatie van de EGF receptor. Naast fosforylatie wordt Eps15 tevens gemodificeerd door een klein eiwit, ubiquitine, dat door bepaalde eiwitten (*ubiquitineringsenzymen*) aan Eps15 wordt "geplakt", een proces dat *ubiquitinering* wordt genoemd. Deze ubiquitinering van Eps15 bleek wel geremd te worden tijdens oxidatieve stress. Tot nog toe is de rol van ubiquitinering in de internalisatie van de EGF receptor niet bekend. Vanuit de literatuur is echter wel bekend dat ubiquitinering van bepaalde eiwitten nodig is voor endocytose van een aantal andere receptoren en ubiquitine zou een "trigger" zijn voor internalisatie. Daarom hebben we aan het eind van hoofdstuk 3 gesuggereerd dat de internalisatie van de EGF receptor mogelijk wordt geremd tijdens oxidatieve stress door remming van de ubiquitinering van eiwitten die betrokken zijn bij EGF receptor internalisatie.

De effecten van oxidatieve stress op de internalisatie van de EGF receptor die in hoofdstuk 3 zijn beschreven, zijn voor een groot gedeelte bestudeerd met radioactief gelabeled EGF. Om de effecten van verscheidene condities en/of stoffen op de internalisatie te meten is het natuurlijk prettiger als dit niet radioactief hoeft te gebeuren. Daarom hebben

we in **hoofdstuk 4** een assay ontwikkeld om op een niet-radioactieve manier de internalisatie van de EGF receptor te meten. Met deze assay werden resultaten behaald die vergelijkbaar waren met resultaten die met radioactiviteit werden gemeten. Daarom is deze nieuw ontwikkelde assay een betrouwbare methode om EGF receptor internalisatie te meten en kan in de toekomst worden gebruikt om de effectiviteit van antioxidanten te testen die bescherming bieden tegen de remming van EGF receptor internalisatie.

Vervolgens zijn we in **hoofdstuk 5** iets dieper op het mechanisme ingegaan dat mogelijk verantwoordelijk is voor de remming van EGF receptor internalisatie. In dit hoofdstuk beschrijven we dat oxidatieve stress (wederom geïnduceerd door waterstofperoxide) niet alleen de ubiquitinering van Eps15 remt, maar ook de ubiquitinering van de EGF receptor zelf. Uit recent gepubliceerde studies is gebleken dat zowel de ubiquitinering van Eps15 als van de EGF receptor plaatsvindt aan de plasma membraan, dus vòòr internalisatie. Dit suggereert een mogelijke rol van ubiquitinering in EGF receptor internalisatie. Vervolgens hebben we onderzocht of de remmende effecten van oxidatieve stress worden opgeheven als cellen niet langer aan deze stress worden blootgesteld. Met andere woorden: er is onderzocht of de effecten *reversibel* zijn en herstellen na verwijdering van waterstofperoxide. Hieruit bleek dat de ubiquitinering van de EGF receptor en Eps15 inderdaad herstelden en dat totaal herstel ± 20 minuten na verwijdering van waterstofperoxide werd bereikt. Ook de internalisatie van de EGF receptor bleek reversibel met een compleet herstel na ± 30 minuten. Omdat de ubiquitinering eerder herstelde dan de internalisatie was dit wederom een aanwijzing dat ubiquitinering mogelijk nodig is voor EGF receptor internalisatie.

Het is bekend dat zuurstofradicalen gemakkelijk *sulfydryl/SH* groepen kunnen oxideren. Vele eiwitten bevatten SH groepen die betrokken kunnen zijn bij o.a. de activatie van deze eiwitten. Oxidatie van deze SH groepen kan daarom een effect hebben op de activiteit van zo'n eiwit en dit kan weer grote gevolgen hebben voor het goed functioneren van de cel. Om te onderzoeken of intacte SH groepen ook een rol spelen in EGF receptor internalisatie hebben we cellen behandeld met een stof die alle SH groepen uitschakelt. Het bleek dat onder deze omstandigheden de internalisatie werd geremd en dit geeft dus aan dat intacte SH groepen nodig zijn voor EGF receptor internalisatie.

Een van de grootste bronnen van SH groepen in de cel is glutathione. Glutathione kan in twee vormen voorkomen, de geoxideerde vorm (*GSSG*) en de gereduceerde (niet-geoxideerde) vorm (*GSH*). Onder normale omstandigheden is de hoeveelheid GSSG in de cel erg laag en is GSH in overmaat aanwezig. De cellulaire ratio GSSG:GSH blijkt ook belangrijk voor het goed verlopen van een groot aantal cellulaire processen en daarom zal de cel ernaar streven om deze ratio zo klein mogelijk te houden. Uit onze studies blijkt de ratio GSSG:GSH tijdens oxidatieve stress behoorlijk snel toe te nemen. Echter, als de oxidatieve stress wordt verwijderd treedt er binnen 10-20 minuten een compleet herstel op

van deze veranderde ratio. Dit herstel gaat dus vooraf aan compleet herstel van EGF receptor internalisatie. Vanuit de literatuur is bekend dat een toename in de ratio GSSG:GSH tijdens oxidatieve stress gepaard kan gaan met een remming van ubiquitinering van eiwitten en dat de activiteit van ubiquitineringsenzymen waarschijnlijk door de GSSG:GSH ratio wordt gereguleerd. Dit komt dus sterk overeen met de resultaten van ons onderzoek.

Alle feiten uit dit hoofdstuk op een rij:

1. Oxidatieve stress (geïnduceerd door waterstofperoxide) verhoogt reversibel de cellulaire GSSG:GSH ratio met een compleet herstel na ± 10-20 minuten
2. Oxidatieve stress remt reversibel de ubiquitinering van de EGF receptor en van Eps15 met herstel na ± 20 minuten
3. Oxidatieve stress remt reversibel EGF receptor internalisatie met compleet herstel na ± 30 minuten
4. Intacte SH groepen zijn nodig voor EGF receptor internalisatie
5. Ubiquitineringsenzymen hebben functionele SH groepen die betrokken zijn bij hun activiteit
6. Ubiquitinering is betrokken bij de internalisatie van een aantal andere receptoren (literatuur)

Deze feiten hebben vervolgens tot het volgende model geleid:

1. Tijdens oxidatieve stress wordt de cellulaire ratio GSSG:GSH verhoogd
2. GSSG reageert vervolgens met SH groepen om de cellulaire ratio GSSG:GSH weer lager te krijgen
3. Reactie van GSSG met SH groepen van ubiquitineringsenzymen leidt tot inactivatie van deze enzymen
4. Hierdoor wordt de ubiquitinering van eiwitten die betrokken zijn bij EGF receptor internalisatie geremd
5. Dit alles leidt uiteindelijk tot een remming van de internalisatie van de EGF receptor

Een schematische tekening van dit model is te zien in de discussie van hoofdstuk 5.
Het is duidelijk dat dit een model is dat in de toekomst verder uitgewerkt en bewezen moet worden. Als er eenmaal bekend is wat het mechanisme precies is, kan er ook doelgericht naar antioxidanten worden gezocht die de cel beschermen tegen de schadelijke effecten van zuurstofradicalen.

De zoektocht naar juiste (combinaties van) antioxidanten/vitamines vraagt erg veel onderzoek en is ingewikkelder dan het op het eerste oog lijkt. Het wordt namelijk steeds meer bekend dat zuurstofradicalen naast hun schadelijke effecten ook juist nodig zijn om de signaaloverdracht in de cel goed te laten verlopen. Het is daarom belangrijk om de effecten van antioxidanten op vele manieren en in vele cellulaire en andere biologische systemen te testen. Zo kunnen bepaalde antioxidanten bescherming bieden tegen bepaalde schadelijke effecten van zuurstofradicalen, maar tegelijkertijd zouden ze andere processen kunnen verstoren die nodig zijn voor het normaal functioneren van de cel. Daarom moet er gezocht

worden naar antioxidanten die de schadelijke effecten van oxidatieve stress voorkomen, zonder het normale functioneren van de cel te beïnvloeden. Daarnaast is het uitermate belangrijk om te onderzoeken wat nu precies de effecten van zuurstofradicalen in de cel zijn. Hierbij moeten we op zoek naar zowel de schadelijke effecten als de “normale” cellulaire processen waar zuurstofradicalen bij betrokken zijn. Op deze manier kunnen we er in de (verre) toekomst misschien achter komen wat er precies gebeurt bij processen als veroudering, het ontstaan van kanker en Alzheimer en kan er doelgericht ingegrepen worden. Misschien komt er dan een tijd dat we allemaal gezond oud kunnen worden!

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Curriculum Vitae

De schrijfster van dit proefschrift werd op 15 december 1973 te Utrecht geboren. De middelbare schoolopleiding werd gevolgd aan het Dr. F.H. de Bruijne Lyceum te Utrecht waar zij in juni 1992 het eindexamen Gymnasium behaalde. In datzelfde jaar begon zij met de studie Biologie aan de Universiteit Utrecht en behaalde in 1993 het propedeutisch examen. In januari 1997 werd het doctoraal examen behaald met als specialisatierichtingen Moleculaire Celbiologie (onder begeleiding van Dr. M.A.G. van der Heyden en Dr. J. Boonstra, Universiteit Utrecht) en Experimentele Longziekten (onder begeleiding van Dr. E. Caldenhoven en Dr. R. de Groot, Universitair Medisch Centrum, Utrecht). In februari 1997 begon zij als Assistent In Opleiding aan de Universiteit Utrecht bij de vakgroep Moleculaire Celbiologie (Prof. Dr. A.J. Verkleij en Prof. Dr. Ir. C.T. Verrips) onder leiding van Dr. J.A. Post en Dr. J. Boonstra. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht.